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[54] **METHOD AND COMPOSITION FOR REGULATING APOPTOSIS**

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[58] **Field of Search** 435/6, 23, 7.8; 530/350, 300

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[57] **ABSTRACT**

This invention provides non-naturally occurring and isolated naturally occurring nucleic acid molecules which encode proteins designated Yama. This invention also provides the polypeptides and proteins encoded by these nucleic acids as well as the purified native polypeptides or proteins. Also provided by this invention is a non-naturally occurring nucleic acid molecule encoding mutant CrmA protein and a dominant inhibitory Yama. Vectors and host cells containing these nucleic acid molecules are further provided. Methods of modulating a cellular function regulated by the Fas receptor pathway in a cell is provided herein. In one aspect, this method comprises introducing into the cell a nucleic acid molecule coding for a gene product having CrmA biological activity such as dominant inhibitory Yama or alternatively, the CrmA gene product. Yama nucleic acid molecules and proteins also can be introduced into the cell to modulate the cellular function regulated by the Fas receptor.

7 Claims, 37 Drawing Sheets

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EMBL Database, Accession No. T10341, Sequence reference HS341, M.B. Soares et al., (Apr. 17, 1994) XP002013935, 1 page total.

YAMA Nucleotide Sequence

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GGCACGAGCG GATGGGTGCT ATTGTGAGGC GGTTGTAGAA GAGTTTCGTG AGTGCTCGCA   60
GCTCATACCT GTGGCTGTGT ATCCGTGGCC ACAGCTGGTT GGCGTCGCCT TGAAATCCCA  120
GGCCGTGAGG AGTTAGCGAG CCCTGCTCAC ACTCGGCGCT CTGGTTTTCG GTGGGTGTGC  180
CCTGCACCTG CCTCTTCCCG CATTCTCATT AATAAAGGTA TCCATGGAGA ACACTGAAAA  240
CTCAGTGGAT TCAAAATCCA TTAAAAATTT GGAACCAAAG ATCATACATG GAAGCGAATC  300
AATGGACTCT GGAATATCCC TGGACAACAG TTATAAAATG GATTATCCTG AGATGGGTTT  360
ATGTATAATA ATTAATAATA AGAATTTTCA TAAAAGCACT GGAATGACAT CTCGGTCTGG  420
TACAGATGTC GATGCAGCAA ACCTCAGGGA AACATTCAGA AACTTGAAAT ATGAAGTCAG  480
GAATAAAAAT GATCTTACAC GTGAAGAAAT TGTGGAATTG ATGCGTGATG TTTCTAAAGA  540
AGATCACAGC AAAAGGAGCA GTTTTGTTTG TGTGCTTCTG AGCCATGGTG AAGAAGGAAT  600
AATTTTTGGA ACAAATGGAC CTGTTGACCT GAAAAAAATA ACAAACTTTT TCAGAGGGGA  660
TCGTTGTAGA AGTCTAACTG GAAAACCCAA ACTTTTCATT ATTCAGGCCT GCCGTGGTAC  720
AGAACTGGAC TGTGGCATTG AGACAGACAG TGGTGTTGAT GATGACATGG CGTGTCATAA  780
AATACCAGTG GAGGCCGACT TCTTGTATGC ATACTCCACA GCACCTGGTT ATTATTCTTG  840
GCGAAATTCA AAGGATGGCT CCTGGTTCAT CCAGTCGCTT TGTGCCATGC TGAAACAGTA  900
TGCCGACAAG CTTGAATTTA TGCACATTCT TACCCGGGTT AACCGAAAGG TGGCAACAGA  960
ATTTGAGTCC TTTTCCTTTG ACGCTACTTT TCATGCAAAG AAACAGATTC CATGTATTGT 1020
TTCCATGCTC ACAAAAGAAC TCTATTTTTA TCACTAAAGA AATGGTTGGT TGGTGGTTTT 1080
TTTTAGTTTG TATGCCAAGT GAGAAGATGG TATATTTGGT ACTGTATTTC CCTCTCATTT 1140
GGGCCTACTC TCATGCTG                                               1158
```

YAMA Peptide Sequence

```
MENTENSVDS KSIKNLEPKI IHGSESMDSG ISLDNSYKMD YPEMGLCIII NNKNFHKSTG   60
MTSRSGTDVD AANLRETFRN LKYEVRNKND LTREEIVELM RDVSKEDHSK RSSFVCVLLS  120
HGEEGIIFGT NGPVDLKKIT NFFRGDRCRS LTGKPKLFII QACRGTELDC GIETDSGVDD  180
DMACHKIPVE ADFLYAYSTA PGYYSWRNSK DGSWFIQSLC AMLKQYADKL EFMHILTRVN  240
RKVATEFESF SFDATFHAKK QIPCIVSMLT KELYFYH                           277
```

FIG. 1

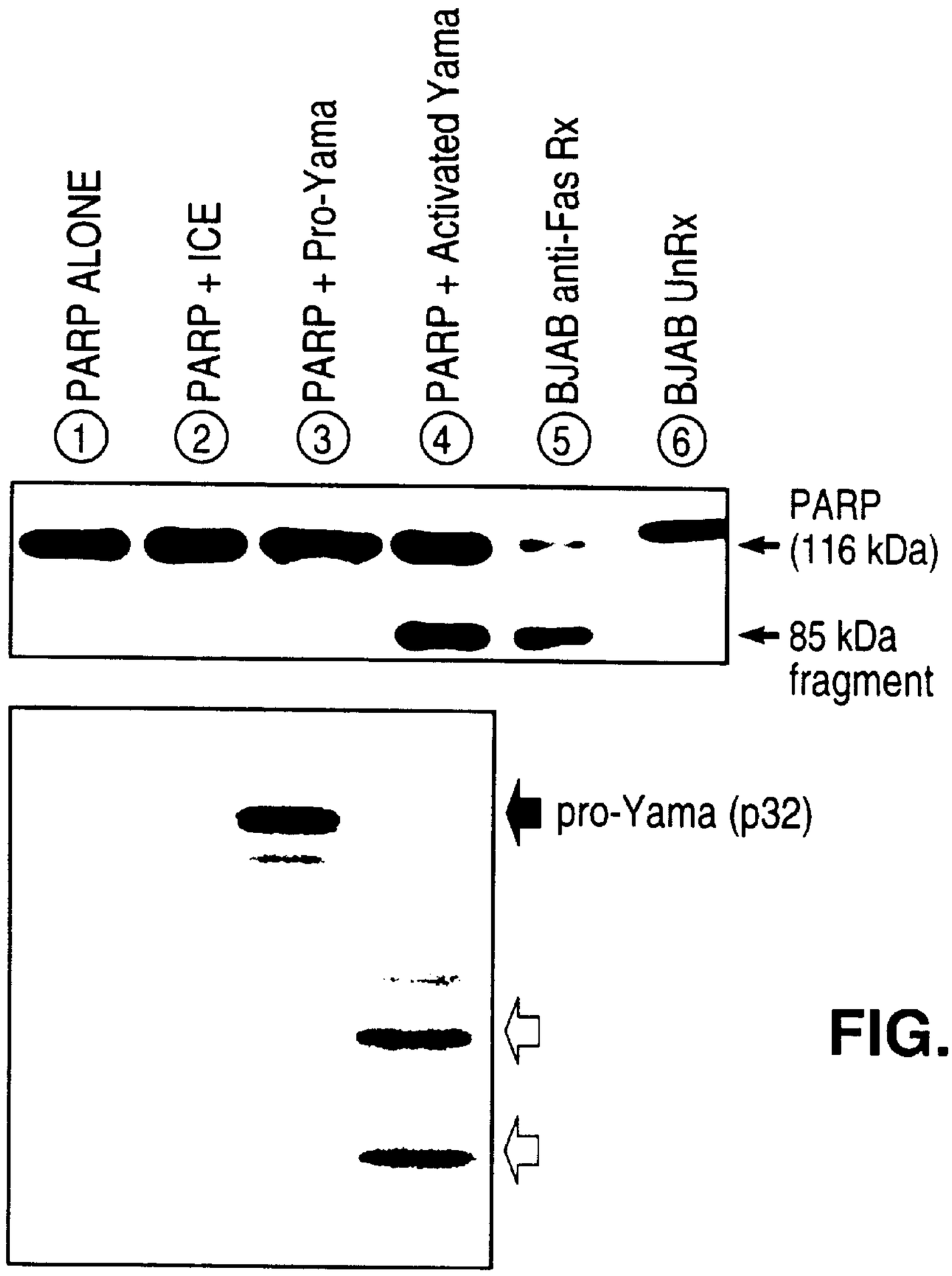
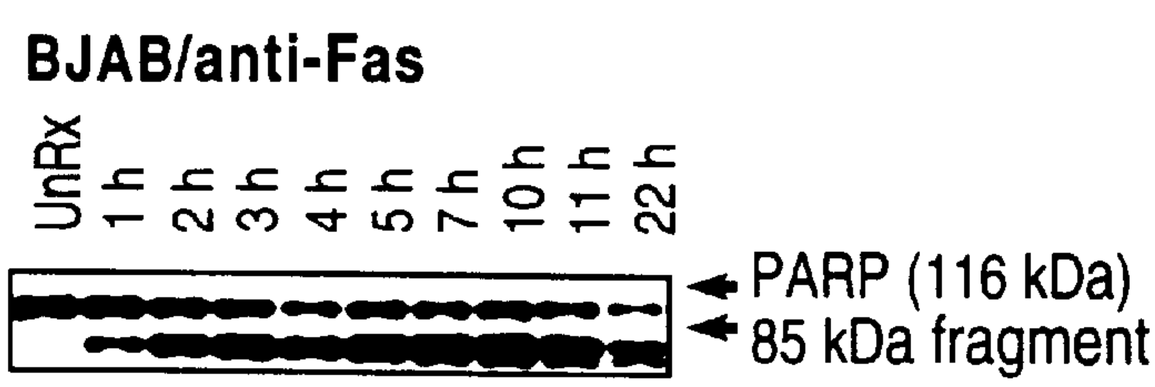

PARP ALONE
PARP + ICE
PARP + Pro-Yama
PARP + Activated Yama
BJAB anti-Fas Rx
BJAB UnRx
1
2
3
4
5
6
PARP
(116 kDa)
85 kDa
fragment
pro-Yama (p32)

FIG. 2A


BJAB/anti-Fas
UnRx
1 h
2 h
3 h
4 h
5 h
7 h
10 h
11 h
22 h
PARP (116 kDa)
85 kDa fragment

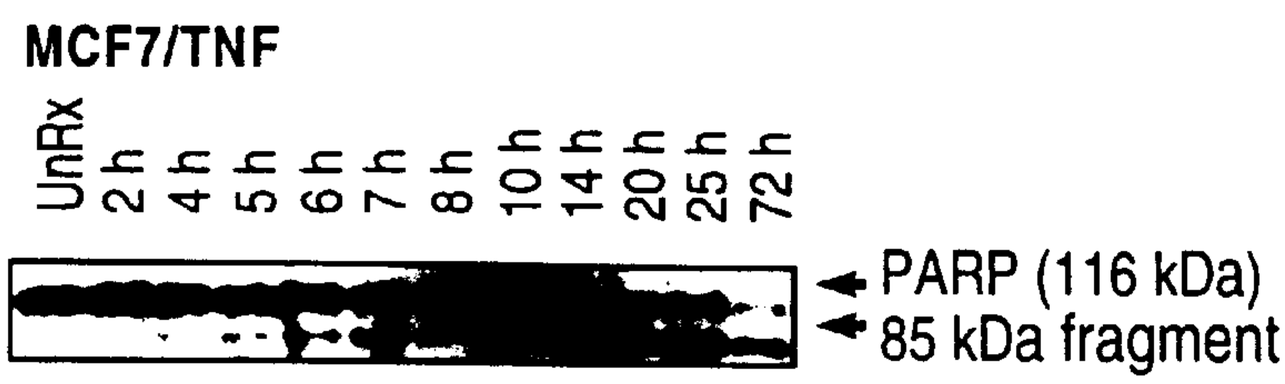

MCF7/TNF
UnRx
2 h
4 h
5 h
6 h
7 h
8 h
10 h
14 h
20 h
25 h
72 h
PARP (116 kDa)
85 kDa fragment

FIG. 2B

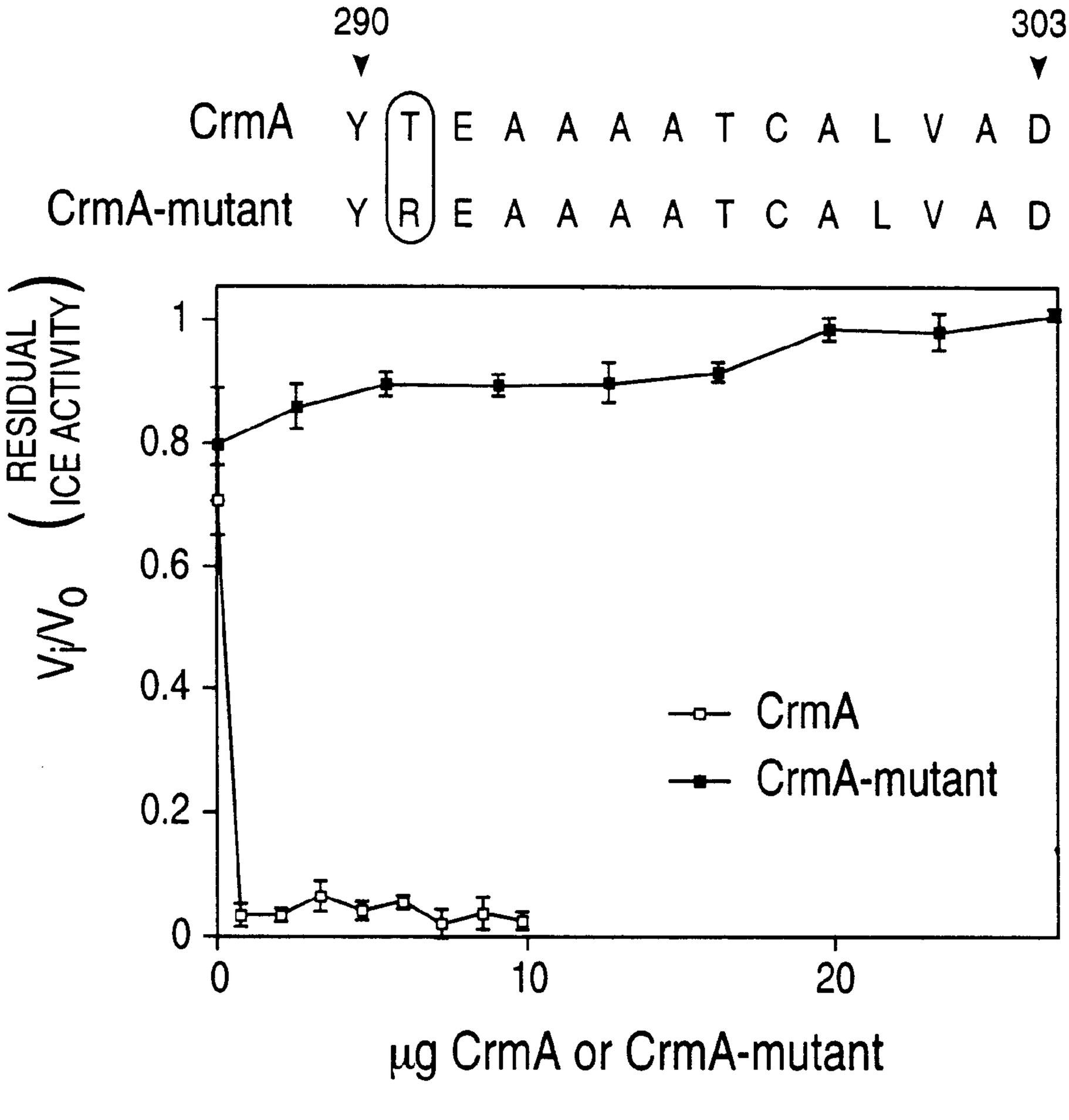

290
303
CrmA Y T E A A A A T C A L V A D
CrmA-mutant Y R E A A A A T C A L V A D
V_i/V_o (RESIDUAL ICE ACTIVITY)
1
0.8
0.6
0.4
0.2
0
0
10
20
CrmA
CrmA-mutant
μg CrmA or CrmA-mutant

FIG. 3A

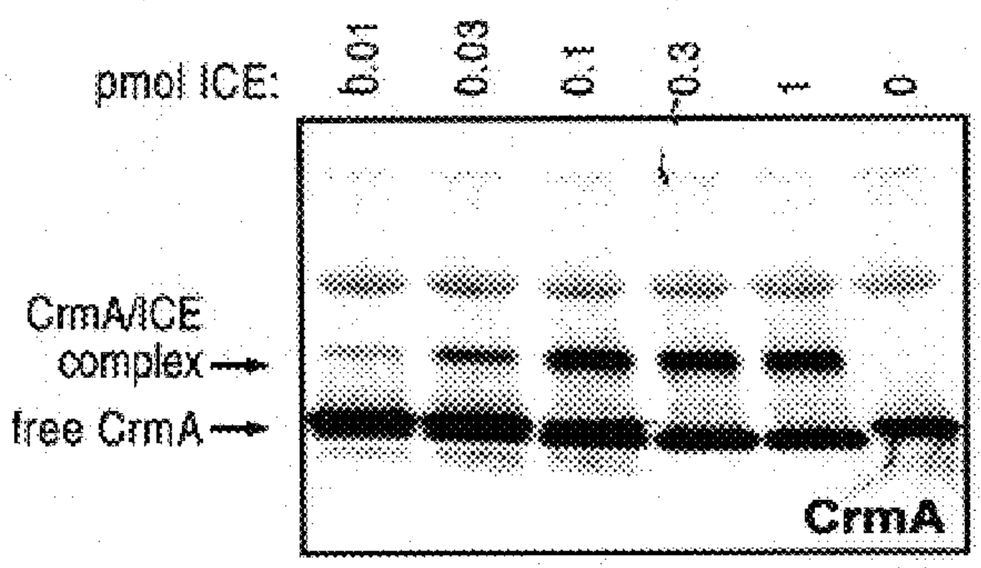

pmol ICE:
0.01
0.03
0.1
0.3
1
0
CrmA/ICE complex
free CrmA
CrmA
free CrmA-mutant
CrmA-mutant

FIG. 3B

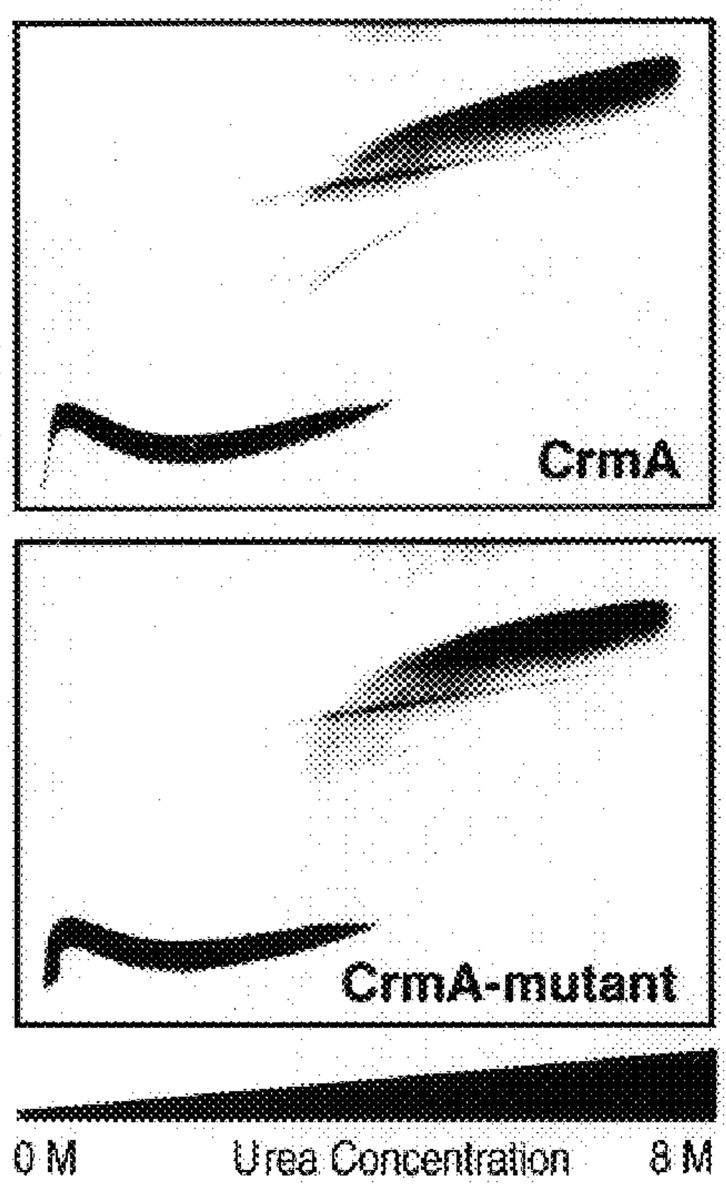

CrmA
CrmA-mutant
0 M
Urea Concentration
8 M

FIG. 3C

FIG. 4A

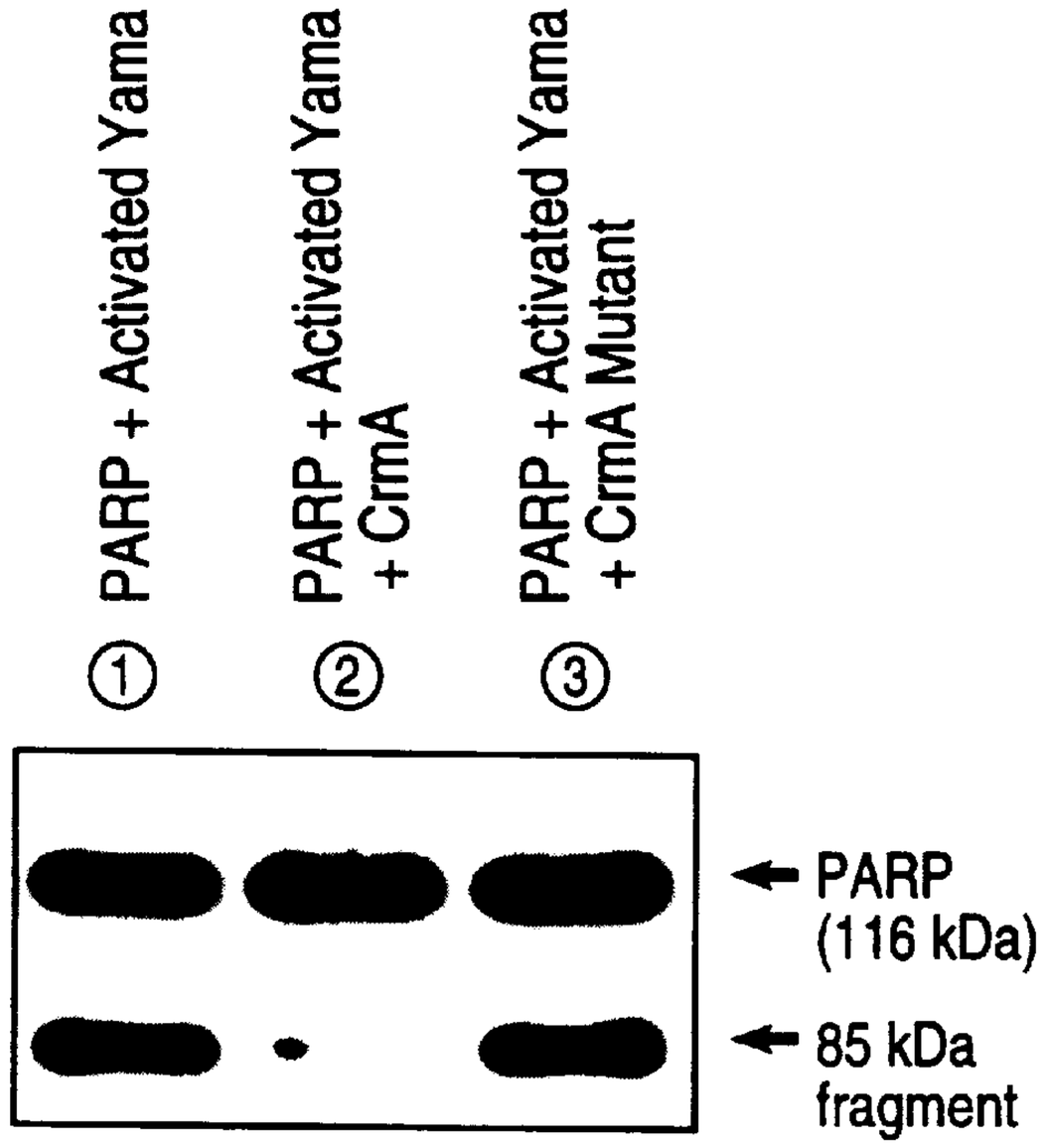
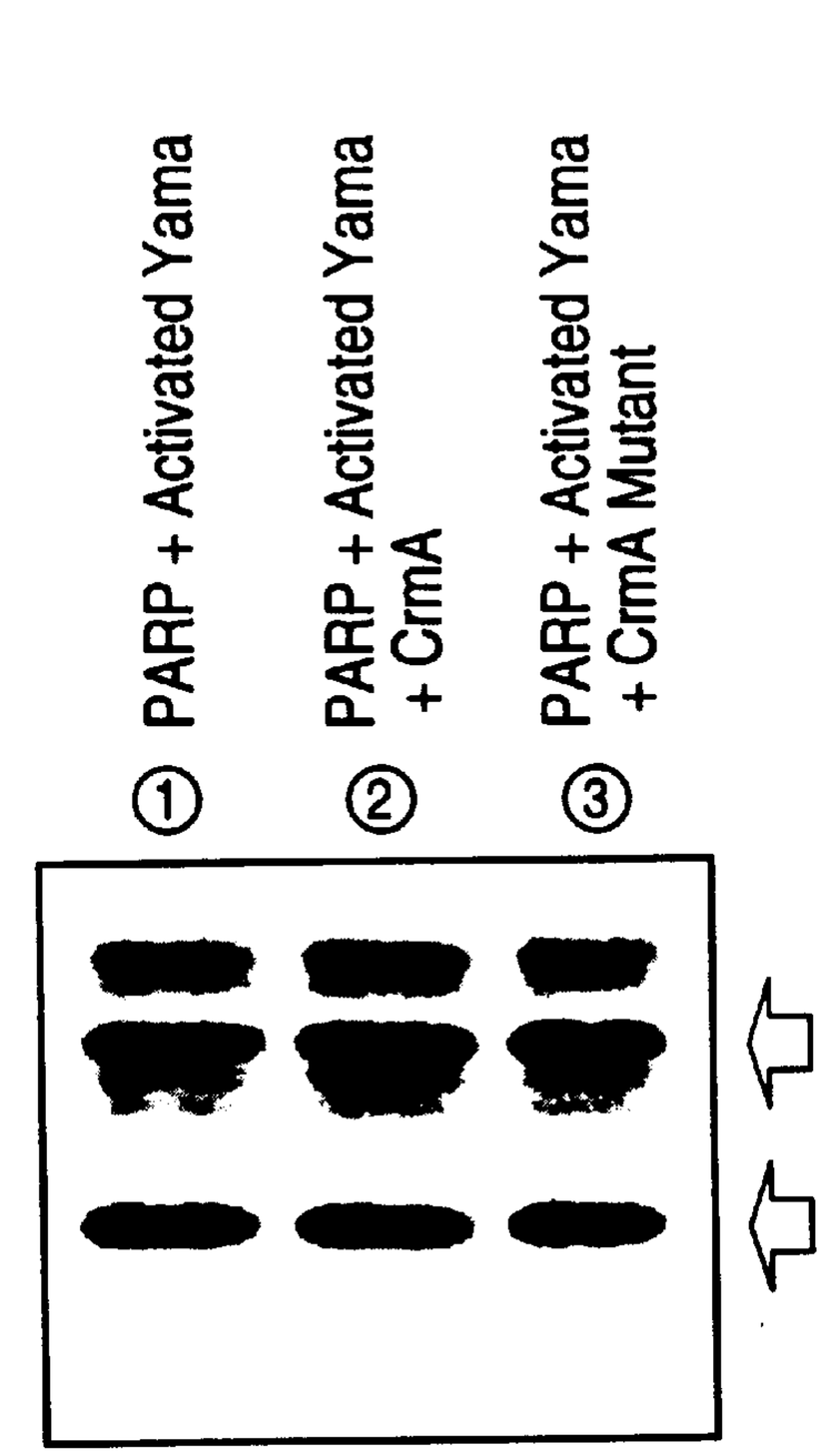

① PARP + Activated Yama
② PARP + Activated Yama + CrmA
③ PARP + Activated Yama + CrmA Mutant
PARP (116 kDa)
85 kDa fragment

FIG. 4B


① PARP + Activated Yama
② PARP + Activated Yama + CrmA
③ PARP + Activated Yama + CrmA Mutant

① pro-Yama + CrmA
② activated Yama + CrmA
③ pro-Yama + mutant CrmA
④ activated Yama + mutant CrmA

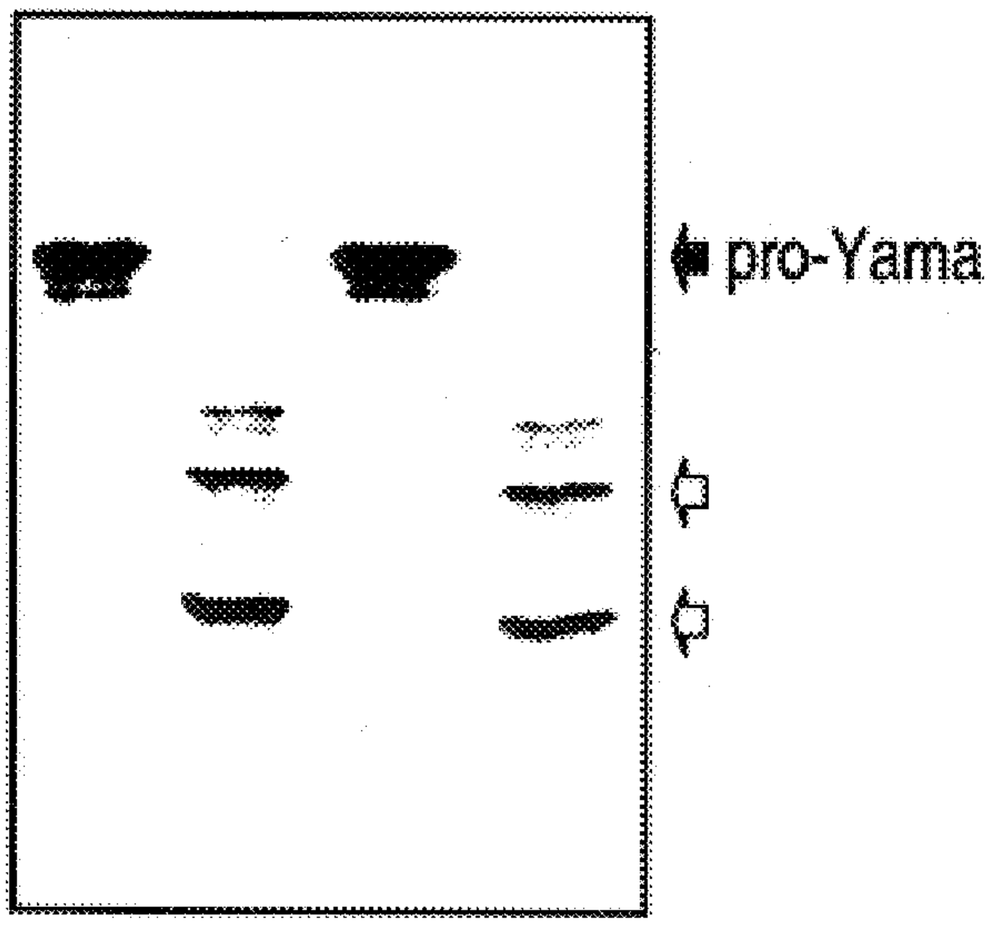


FIG. 5A

① pro-Yama + CrmA
② activated Yama + CrmA
③ pro-Yama + mutant CrmA
④ activated Yama + mutant CrmA

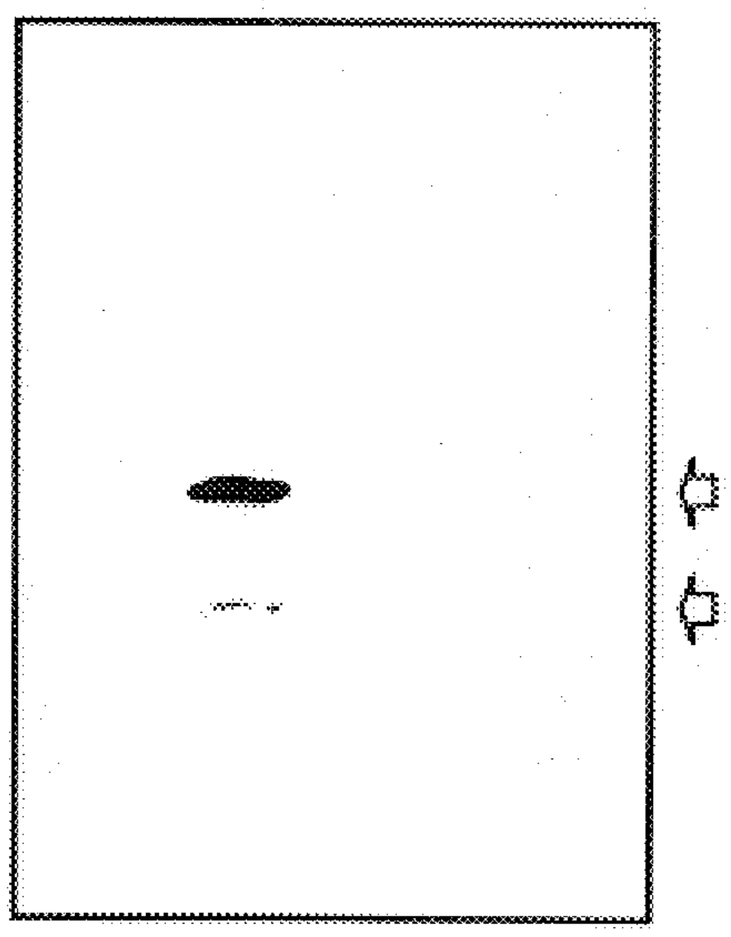


FIG. 5B

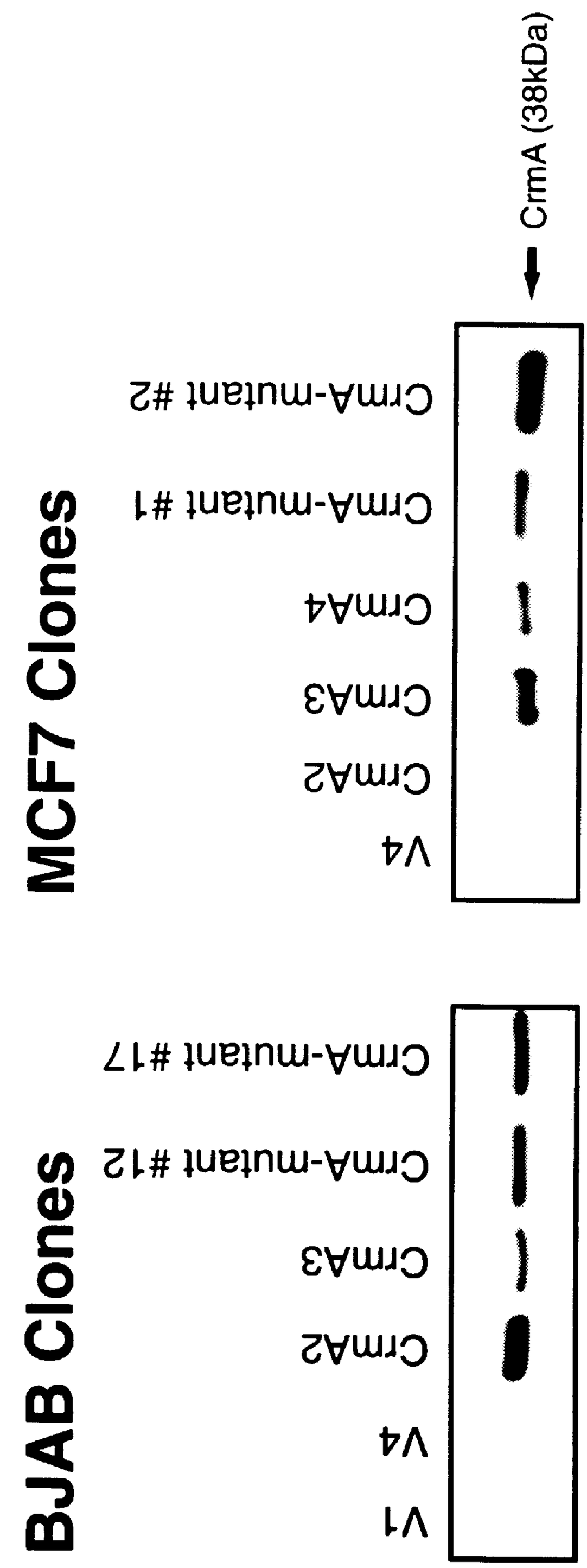

CrmA (38kDa)
MCF7 Clones
V4
CrmA2
CrmA3
CrmA4
CrmA-mutant #1
CrmA-mutant #2
BJAB Clones
V1
V4
CrmA2
CrmA3
CrmA-mutant #12
CrmA-mutant #17

FIG. 6A

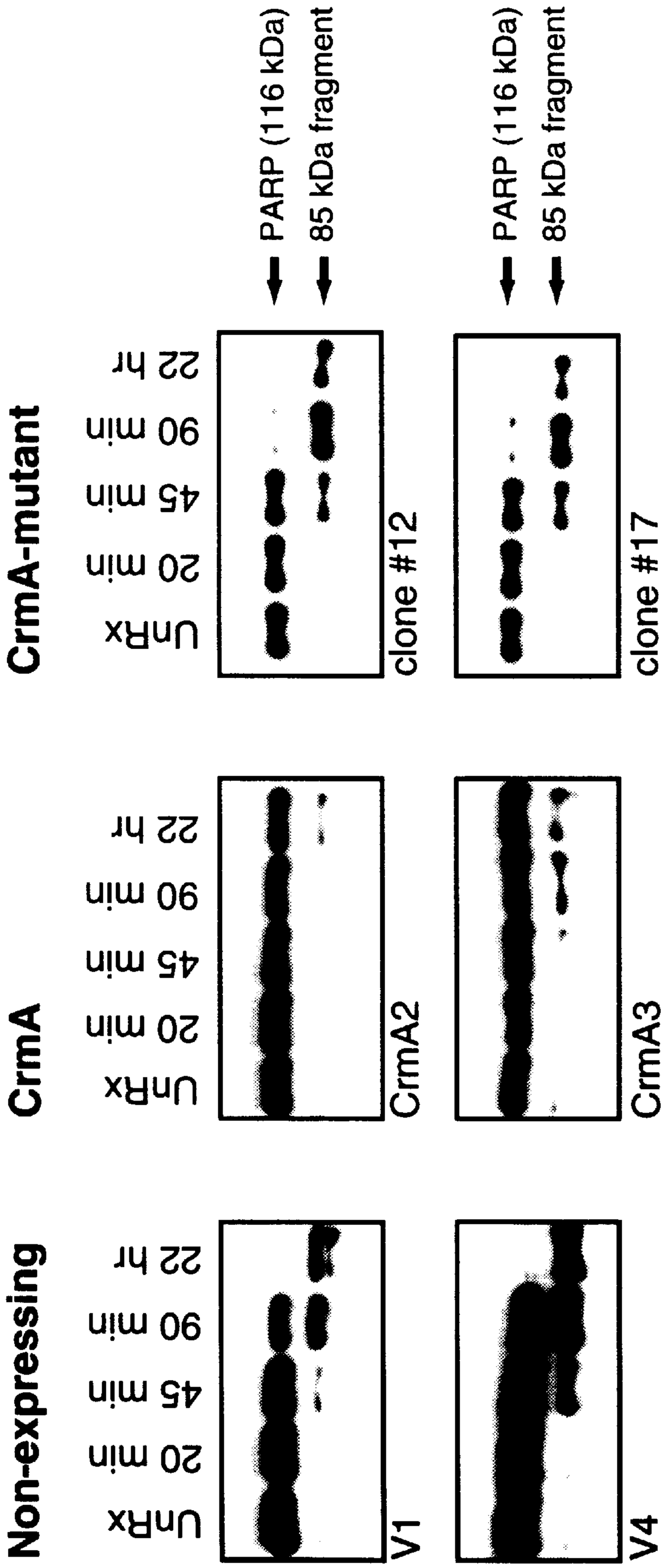

CrmA-mutant
UnRx
20 min
45 min
90 min
22 hr
clone #12
clone #17
PARP (116 kDa)
85 kDa fragment
CrmA
CrmA2
CrmA3
Non-expressing
V1
V4

FIG. 6B

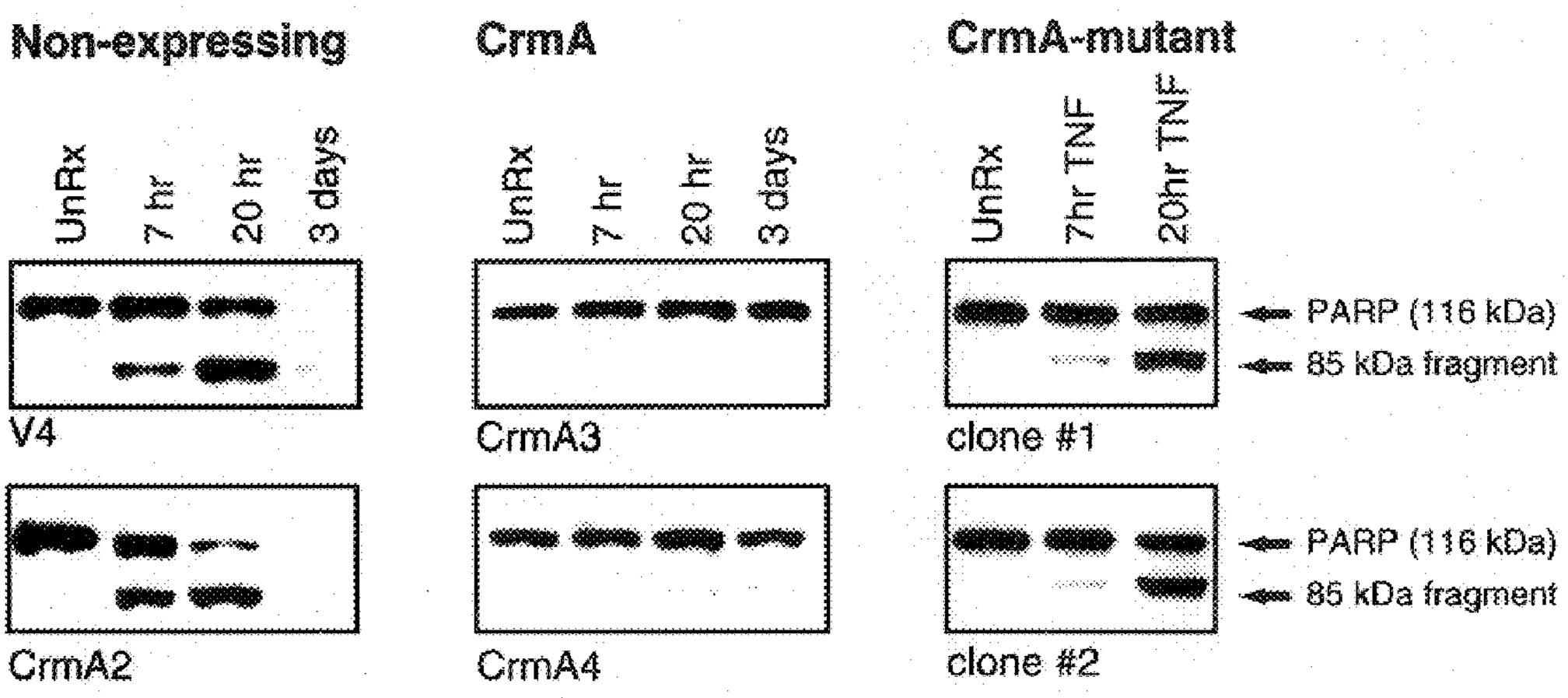

Non-expressing
UnRx
7 hr
20 hr
3 days
V4
CrmA2
CrmA
UnRx
7 hr
20 hr
3 days
CrmA3
CrmA4
CrmA-mutant
UnRx
7hr TNF
20hr TNF
clone #1
clone #2
PARP (116 kDa)
85 kDa fragment
PARP (116 kDa)
85 kDa fragment

FIG. 6C

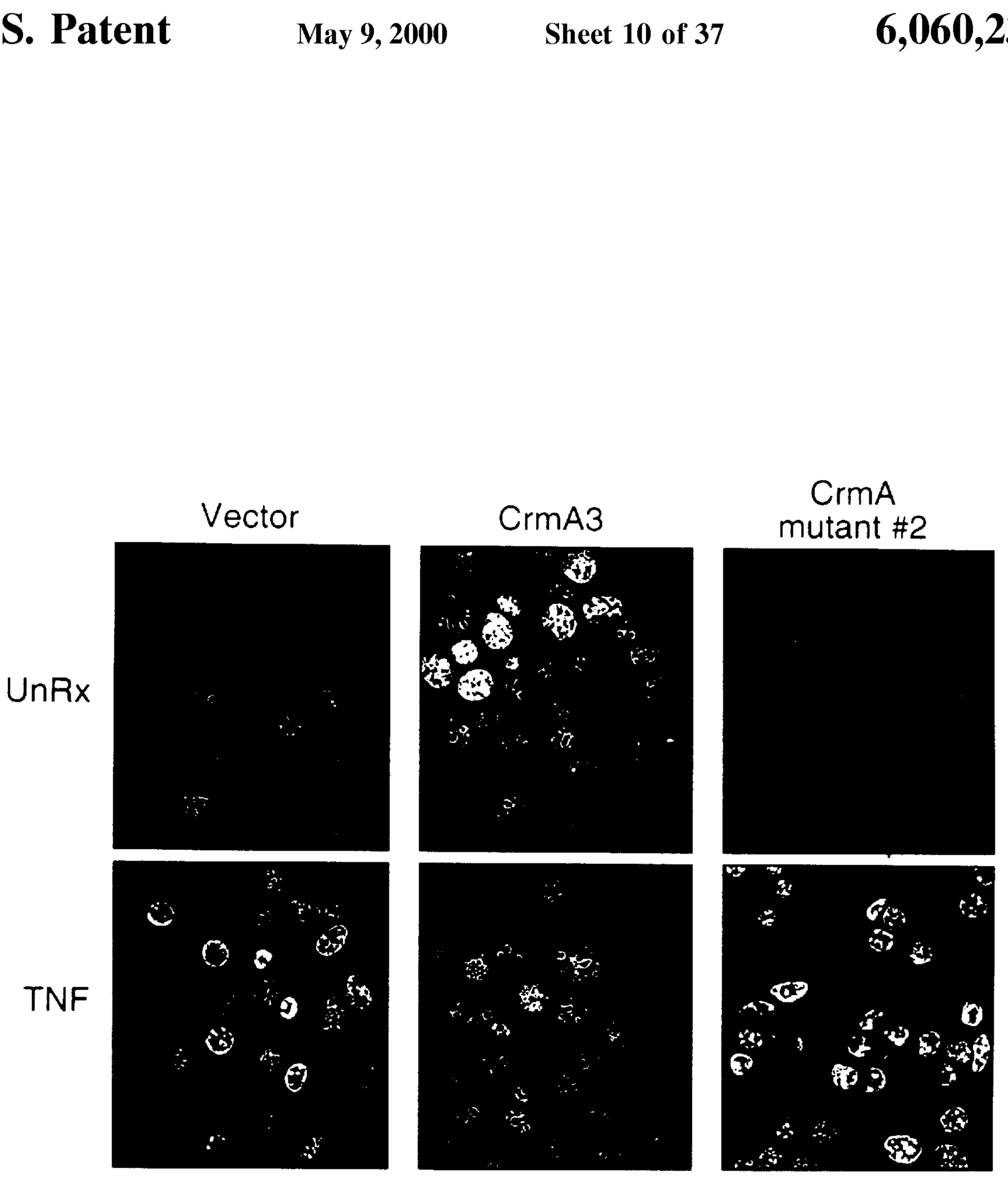


FIG. 7A

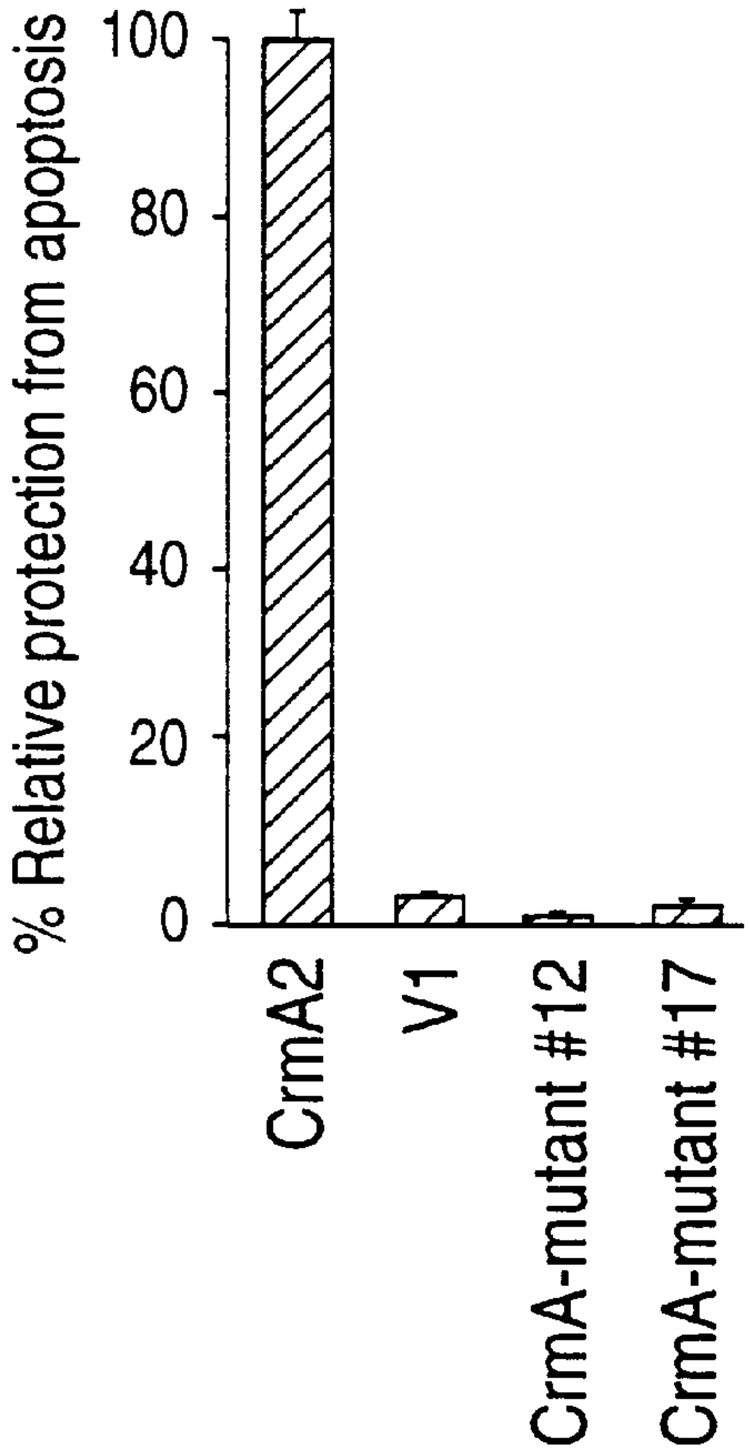

% Relative protection from apoptosis
100
80
60
40
20
0
CrmA2
V1
CrmA-mutant #12
CrmA-mutant #17

FIG. 7B

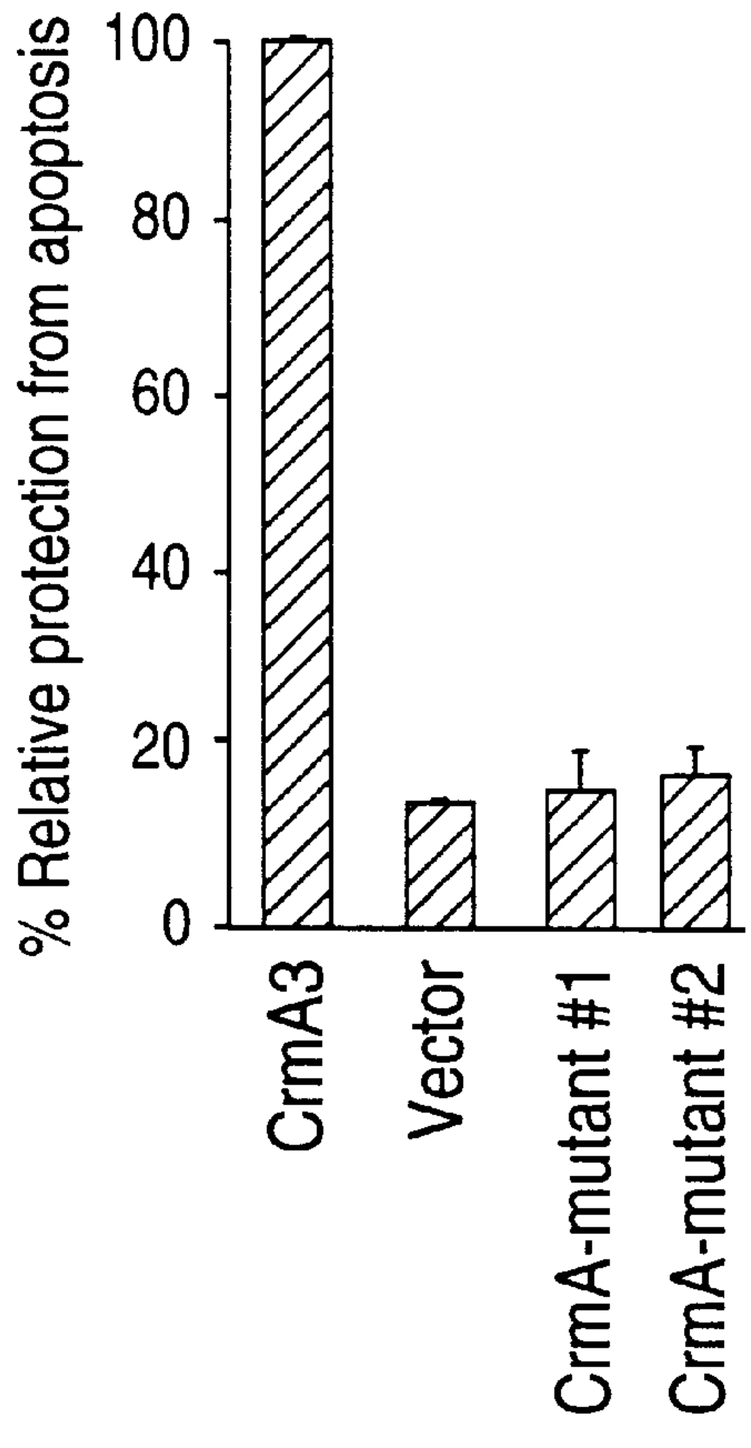

% Relative protection from apoptosis
100
80
60
40
20
0
CrmA3
Vector
CrmA-mutant #1
CrmA-mutant #2

FIG. 7C

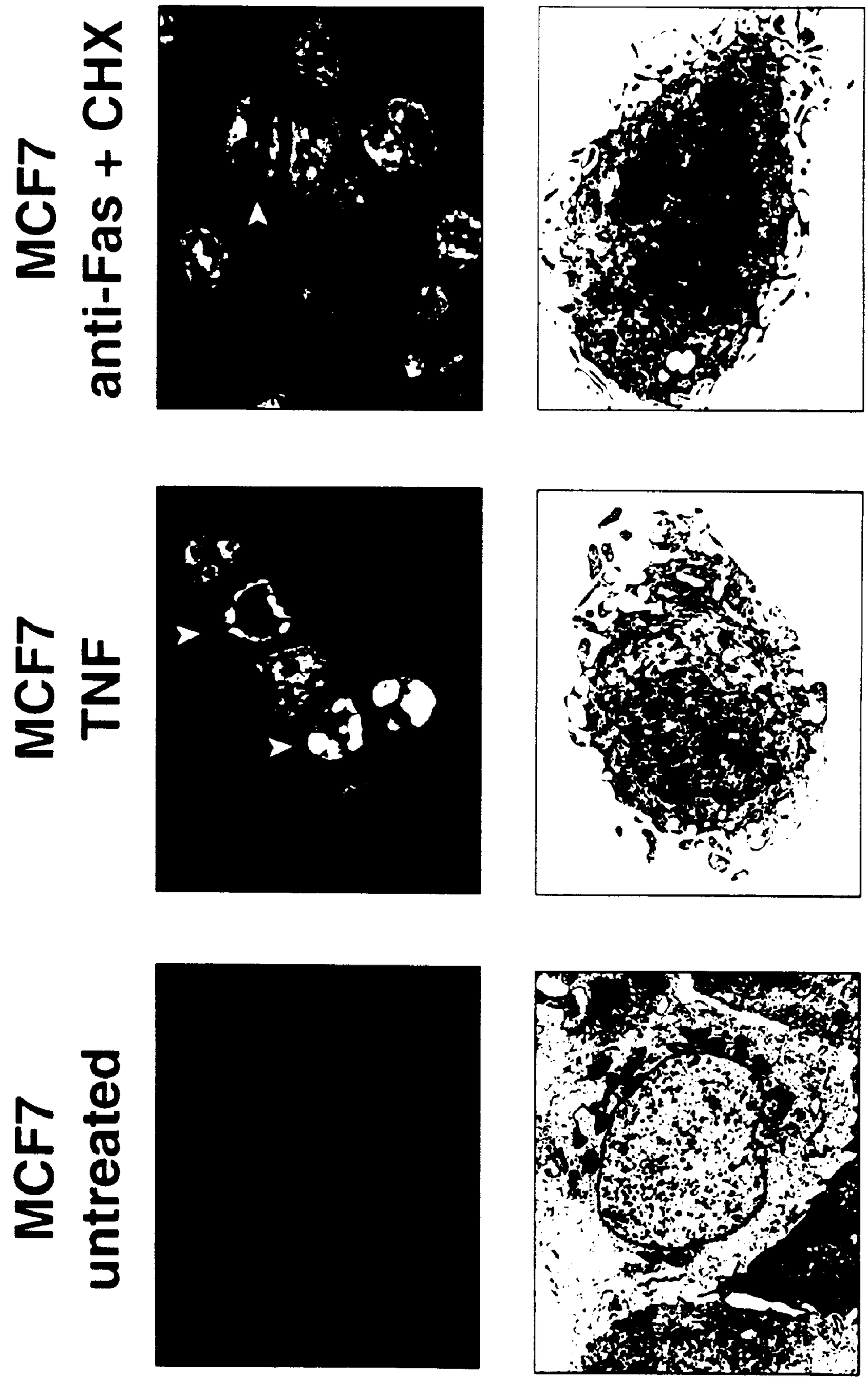


FIG. 8A

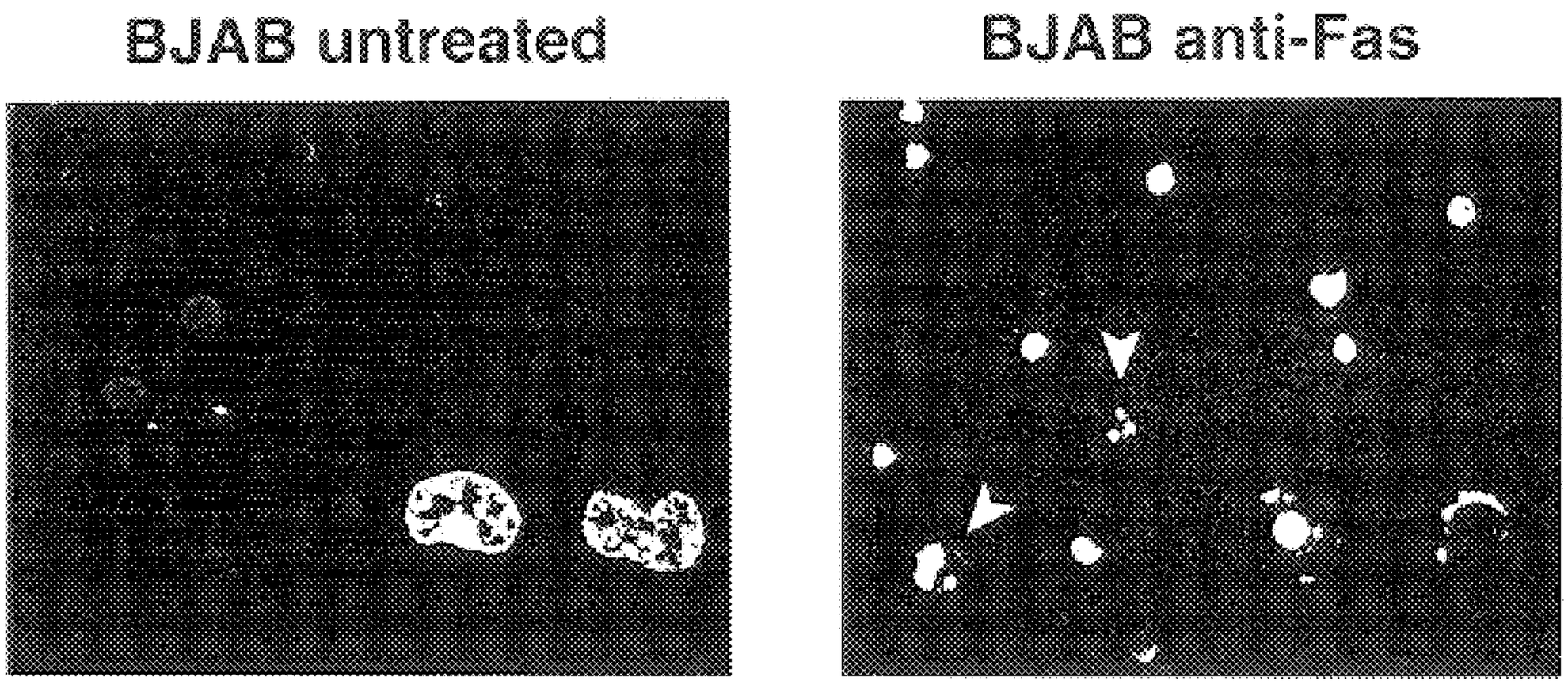


FIG. 8B

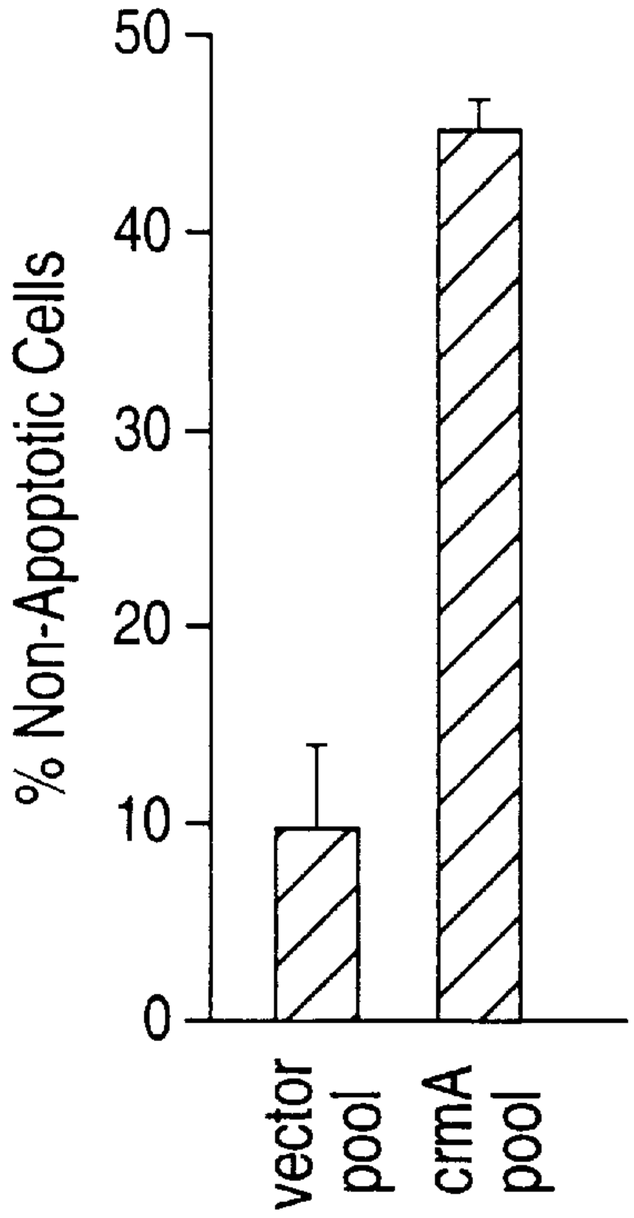


FIG. 9A

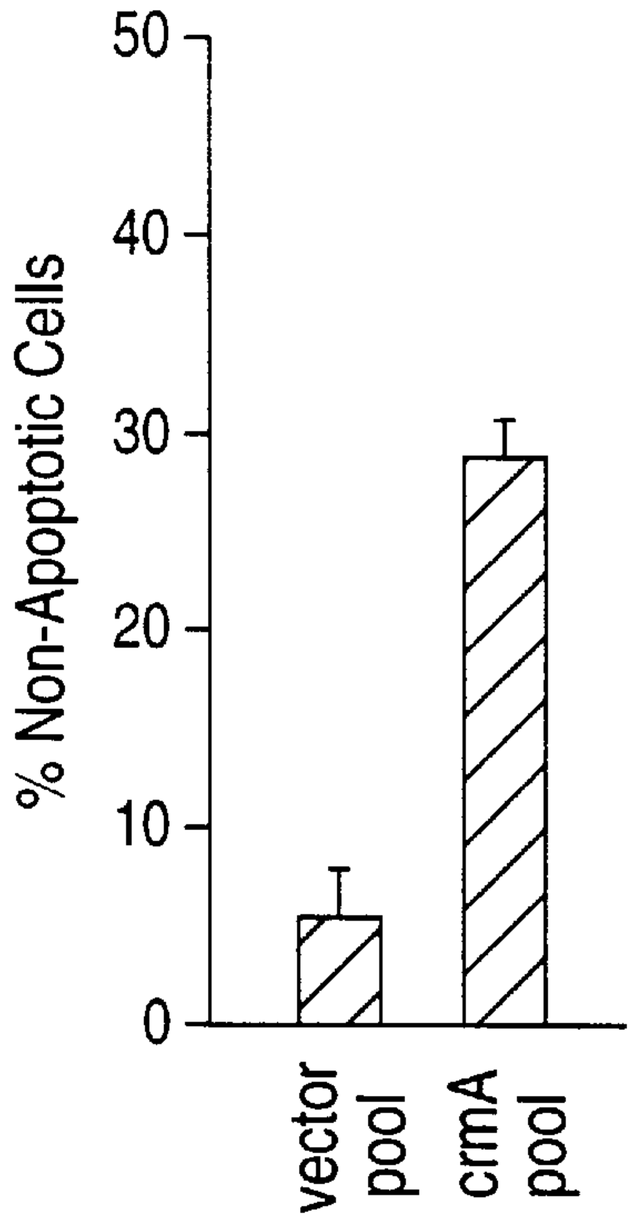


FIG. 9B

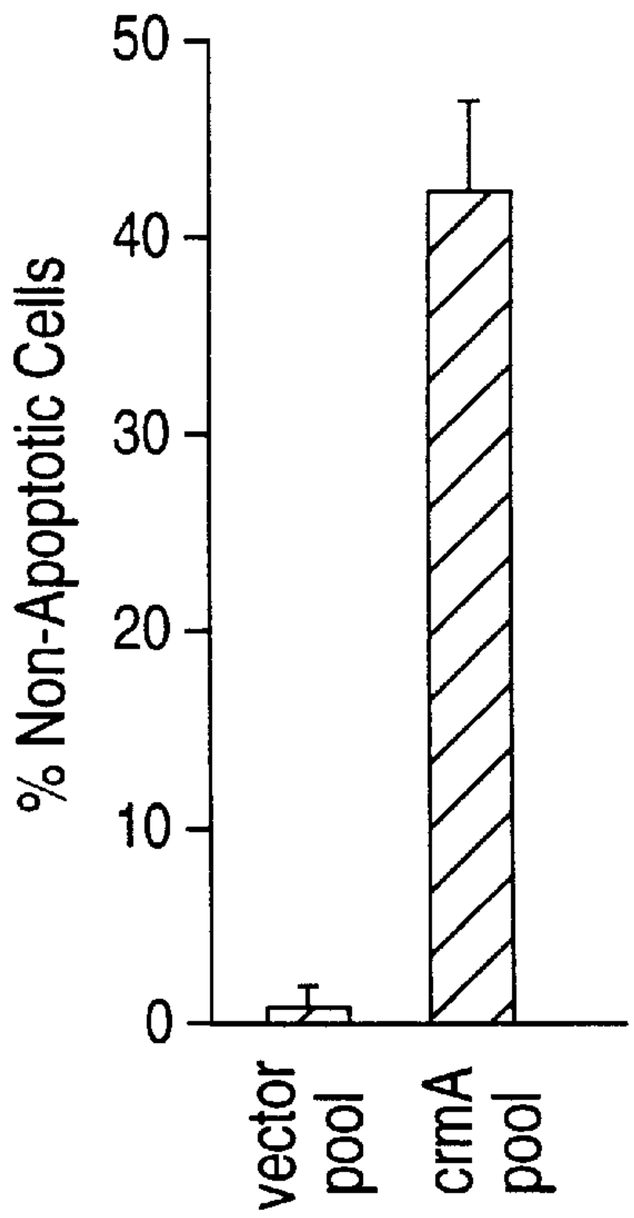


FIG. 9C

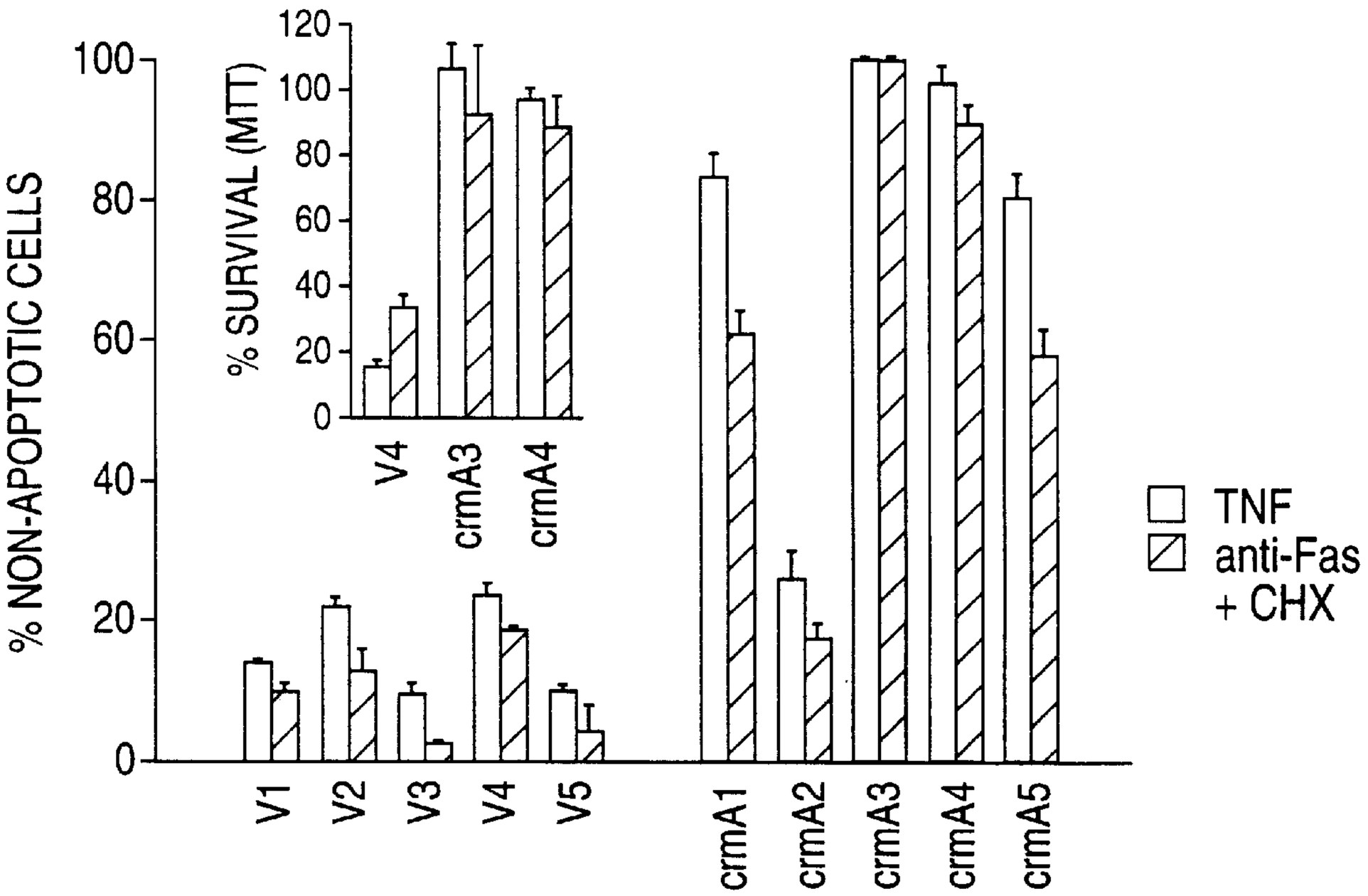

% NON-APOPTOTIC CELLS
100
80
60
40
20
0
% SURVIVAL (MTT)
120
100
80
60
40
20
0
V4
crmA3
crmA4
V1
V2
V3
V4
V5
crmA1
crmA2
crmA3
crmA4
crmA5
TNF
anti-Fas
+ CHX

FIG. 10A

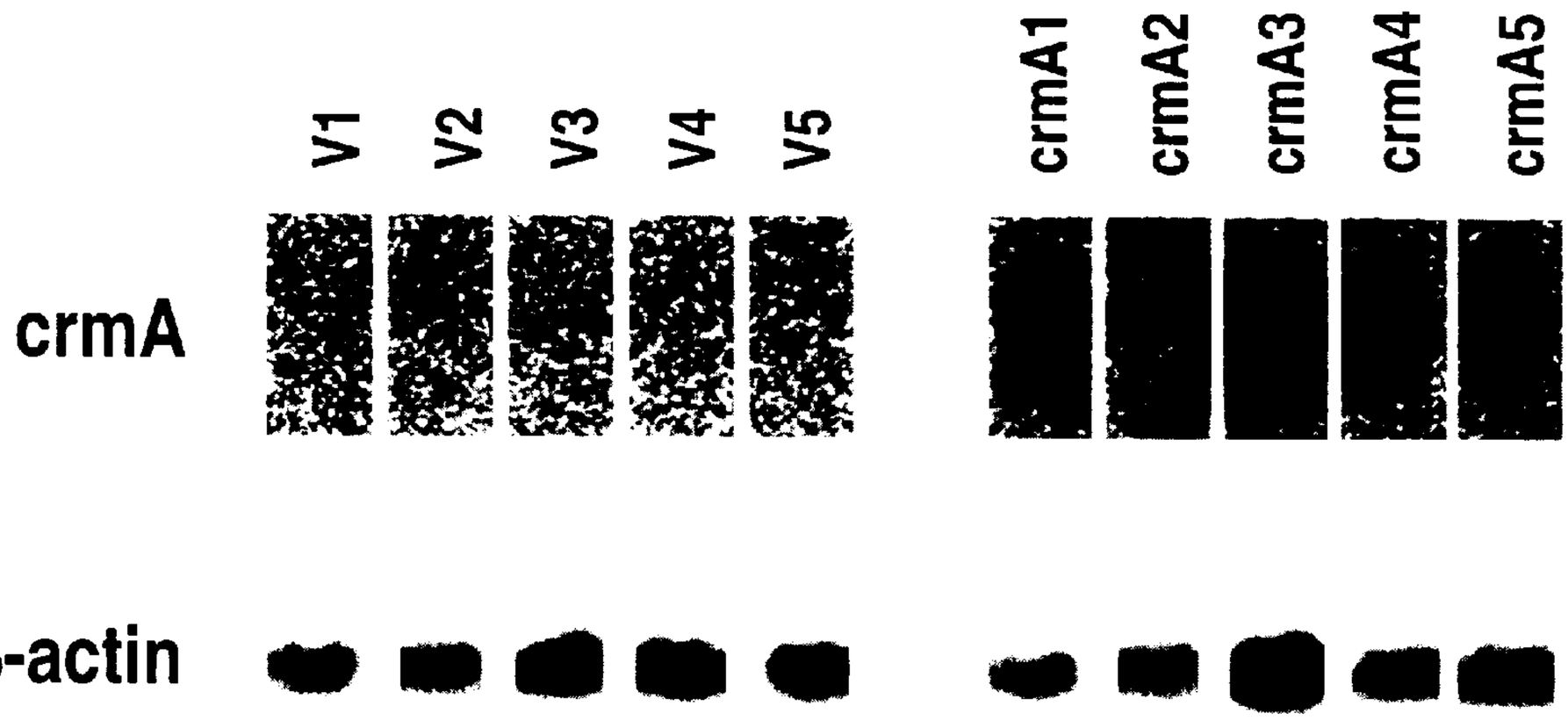


FIG. 10B

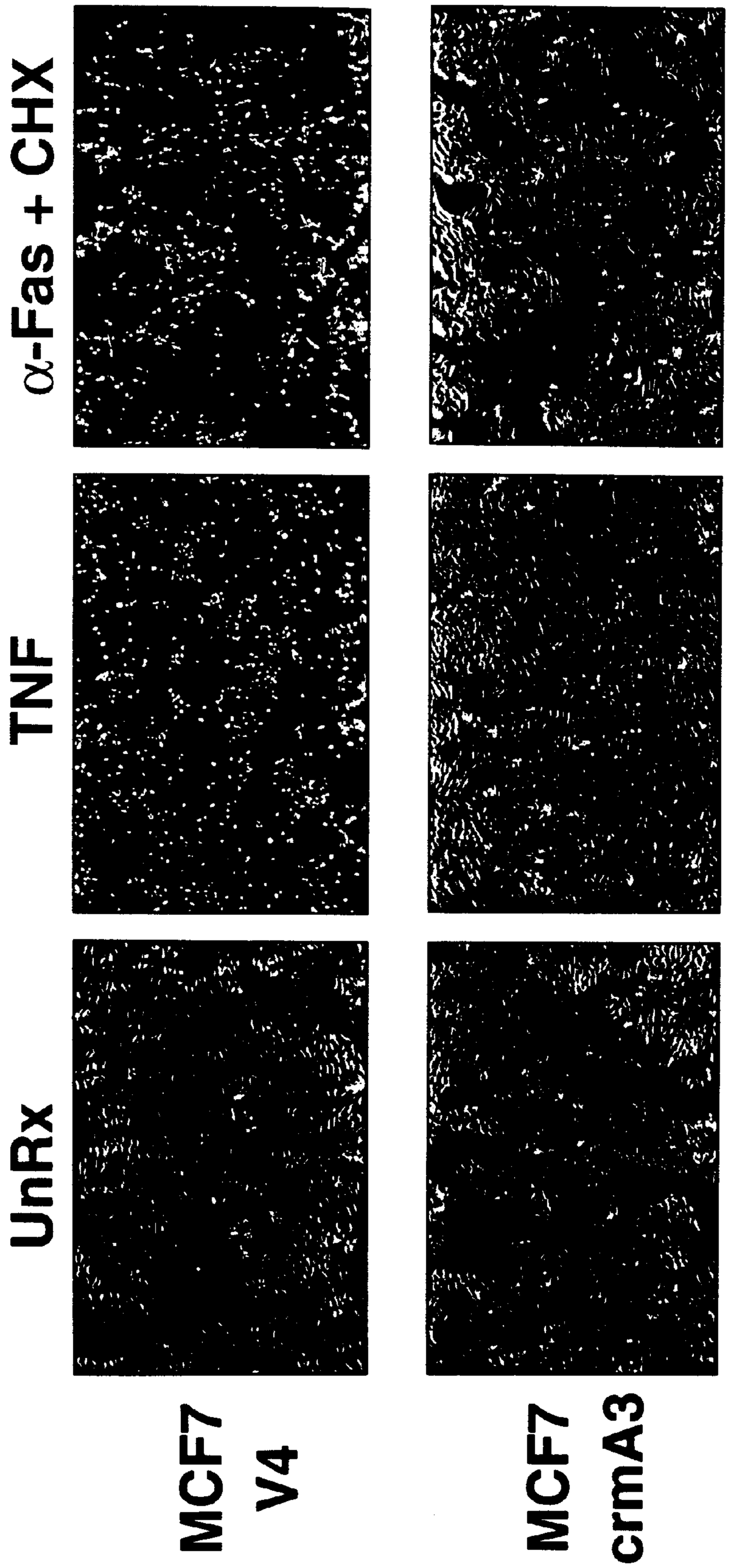


FIG. 10C

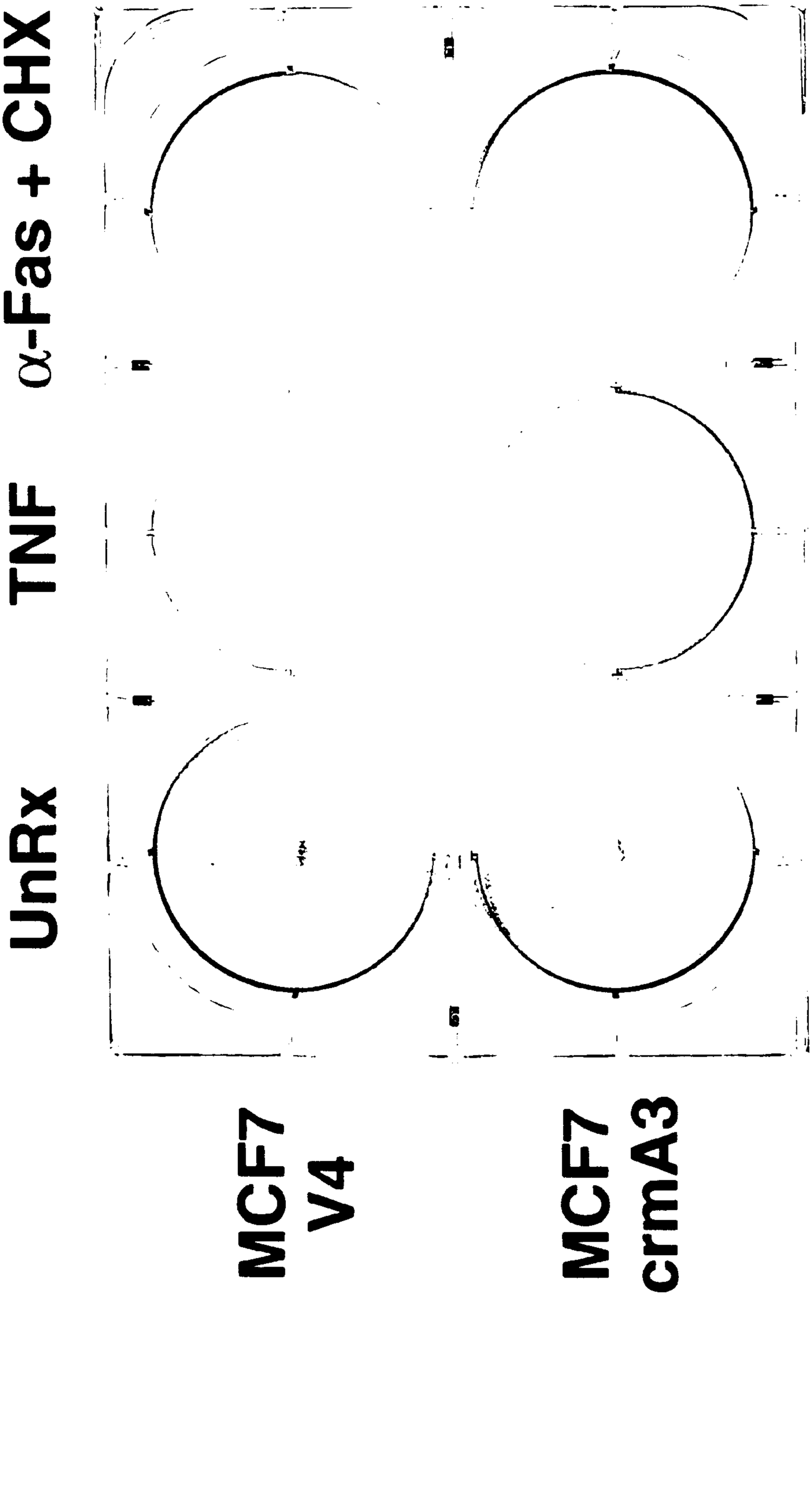


FIG. 10D

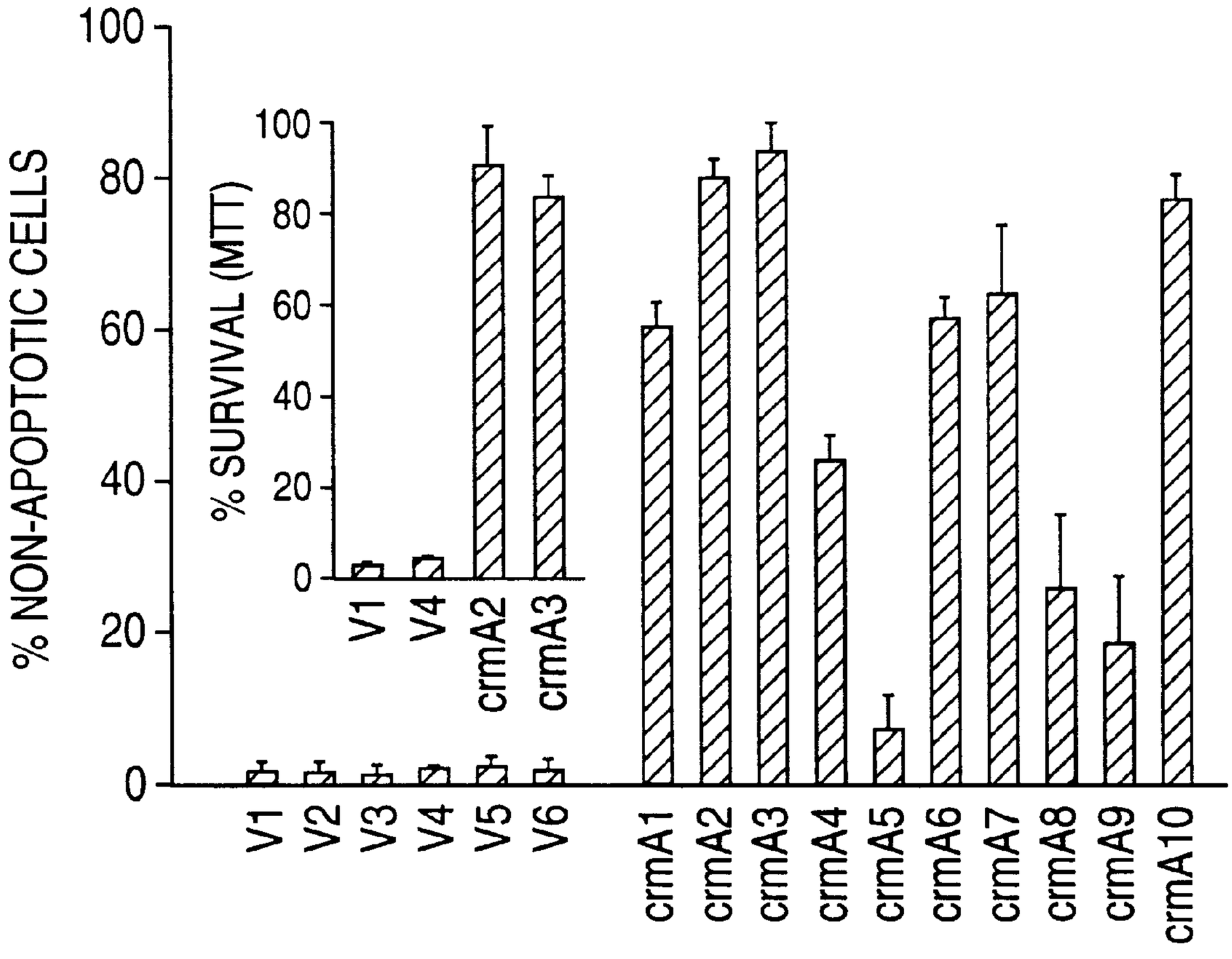

% NON-APOPTOTIC CELLS
100
80
60
40
20
0
V1
V2
V3
V4
V5
V6
% SURVIVAL (MTT)
100
80
60
40
20
0
V1
V4
crmA2
crmA3
crmA1
crmA2
crmA3
crmA4
crmA5
crmA6
crmA7
crmA8
crmA9
crmA10

FIG. 11A

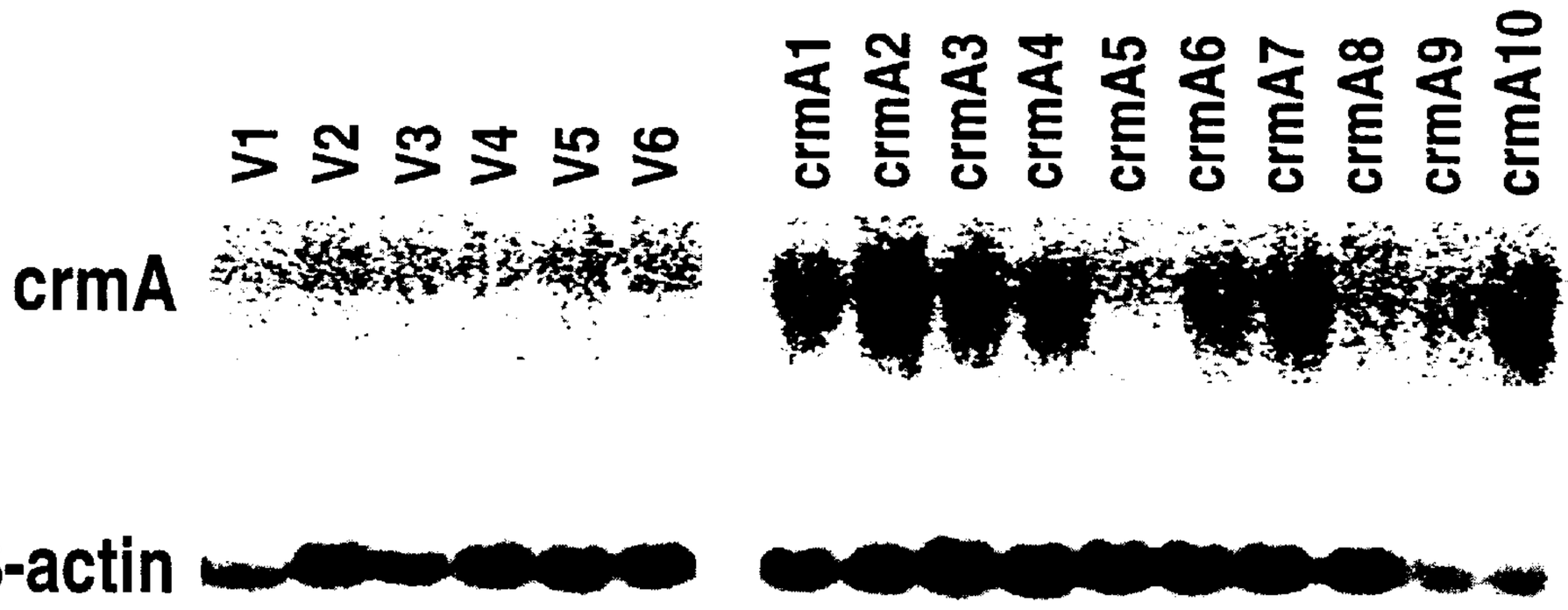

V1
V2
V3
V4
V5
V6
crmA1
crmA2
crmA3
crmA4
crmA5
crmA6
crmA7
crmA8
crmA9
crmA10
crmA
β-actin

FIG. 11B

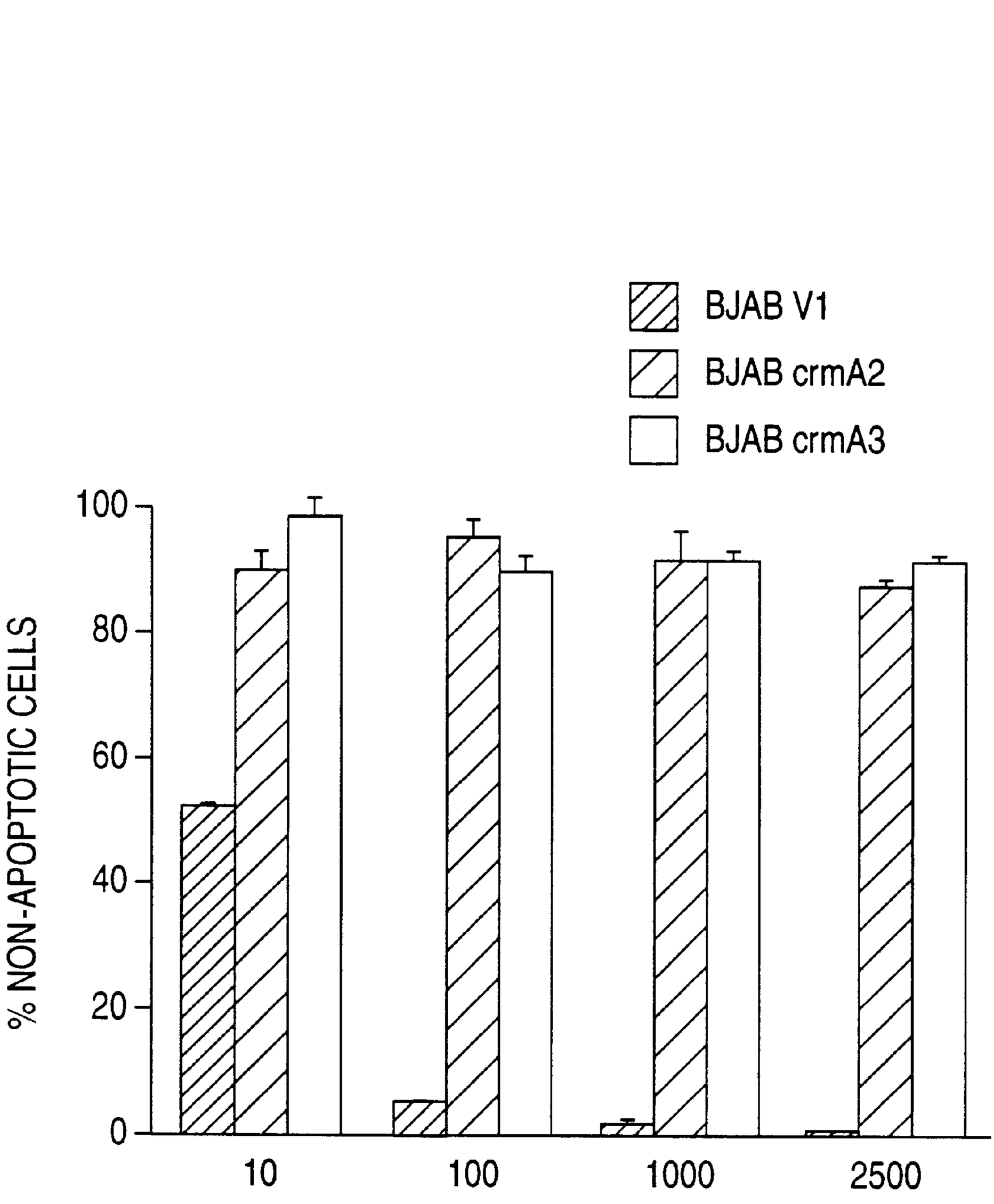

BJAB V1
BJAB crmA2
BJAB crmA3
% NON-APOPTOTIC CELLS
100
80
60
40
20
0
10
100
1000
2500
ng/ml anti-Fas

FIG. 11C

```
TCCATGGAAG AACGAAAGTA GTATAAAAGT AATAAAACAA AAAAAAGAAT ATAAAAAATT    60
TATAGCCACT TTCTTTGAGG ACTGTTTTCC TGAAGGAAAT GAACCTCTGG AATTAGTTAG   120
ATATATAGAA TTAGTATACA CGCTAGATTA TTCTCAAACT CCTAATTATG ACAGACTACG   180
TAGACTGTTT ATACAAGATT GAAAATATAT TTCTTTTTAT TGAGTGGTGG TAGTTACGGA   240
TATCTAATAT TAATATTAGA CTATCTCTAT CGTCACACAA CAAAATCGAT TGCC ATG      297
                                                           Met
                                                            1

GAT ATC TTC AGG GAA ATC GCA TCT TCT ATG AAA GGA GAG AAT GTA TTC      345
Asp Ile Phe Arg Glu Ile Ala Ser Ser Met Lys Gly Glu Asn Val Phe
                5                  10                  15

ATT TCT CCA CCG TCA ATC TCG TCA GTA TTG ACA ATA CTG TAT TAT GGA      393
Ile Ser Pro Pro Ser Ile Ser Ser Val Leu Thr Ile Leu Tyr Tyr Gly
            20                  25                  30

GCT AAT GGA TCC ACT GCT GAA CAG CTA TCA AAA TAT GTA GAA AAG GAG      441
Ala Asn Gly Ser Thr Ala Glu Gln Leu Ser Lys Tyr Val Glu Lys Glu
        35                  40                  45

GCG GAC AAG AAT AAG GAT GAT ATC TCA TTC AAG TCC ATG AAT AAA GTA      489
Ala Asp Lys Asn Lys Asp Asp Ile Ser Phe Lys Ser Met Asn Lys Val
    50                  55                  60                  65

TAT GGG CGA TAT TCT GCA GTG TTT AAA GAT TCC TTT TTG AGA AAA ATT      537
Tyr Gly Arg Tyr Ser Ala Val Phe Lys Asp Ser Phe Leu Arg Lys Ile
                70                  75                  80

GGA GAT AAT TTC CAA ACT GTT GAC TTC ACT GAT TGT CGC ACT GTA GAT      585
Gly Asp Asn Phe Gln Thr Val Asp Phe Thr Asp Cys Arg Thr Val Asp
            85                  90                  95

GCG ATC AAC AAG TGT GTT GAT ATC TTC ACT GAG GGG AAA ATT AAT CCA      633
Ala Ile Asn Lys Cys Val Asp Ile Phe Thr Glu Gly Lys Ile Asn Pro
        100                 105                 110

CTA TTG GAT GAA CCA TTG TCT CCA GAT ACC TGT CTC CTA GCA ATT AGT      681
Leu Leu Asp Glu Pro Leu Ser Pro Asp Thr Cys Leu Leu Ala Ile Ser
    115                 120                 125

GCC GTA TAC TTT AAA GCA AAA TGG TTG ATG CCA TTT GAA AAG GAA TTT      729
Ala Val Tyr Phe Lys Ala Lys Trp Leu Met Pro Phe Glu Lys Glu Phe
130                 135                 140                 145

ACC AGT GAT TAT CCC TTT TAC GTA TCT CCA ACG GAA ATG GTA GAT GTA      777
Thr Ser Asp Tyr Pro Phe Tyr Val Ser Pro Thr Glu Met Val Asp Val
                150                 155                 160

AGT ATG ATG TCT ATG TAC GGC GAG GCA TTT AAT CAC GCA TCT GTA AAA      825
Ser Met Met Ser Met Tyr Gly Glu Ala Phe Asn His Ala Ser Val Lys
            165                 170                 175
```

FIG. 12A

```
GAA TCA TTC GGC AAC TTT TCA ATC ATA GAA CTG CCA TAT GTT GGA GAT     873
Glu Ser Phe Gly Asn Phe Ser Ile Ile Glu Leu Pro Tyr Val Gly Asp
        180                 185                 190

ACT AGT ATG GTG GTA ATT CTT CCA GAC AAT ATT GAT GGA CTA GAA TCC     921
Thr Ser Met Val Val Ile Leu Pro Asp Asn Ile Asp Gly Leu Glu Ser
    195                 200                 205

ATA GAA CAA AAT CTA ACA GAT ACA AAT TTT AAG AAA TGG TGT GAC TCT     969
Ile Glu Gln Asn Leu Thr Asp Thr Asn Phe Lys Lys Trp Cys Asp Ser
210                 215                 220                 225

ATG GAT GCT ATG TTT ATC GAT GTG CAC ATT CCC AAG TTT AAG GTA ACA    1017
Met Asp Ala Met Phe Ile Asp Val His Ile Pro Lys Phe Lys Val Thr
                230                 235                 240

GGC TCG TAT AAT CTG GTG GAT GCG CTA GTA AAG TTG GGA CTG ACA GAG    1065
Gly Ser Tyr Asn Leu Val Asp Ala Leu Val Lys Leu Gly Leu Thr Glu
            245                 250                 255

GTG TTC GGT TCA ACT GGA GAT TAT AGC AAT ATG TGT AAT TCA GAT GTG    1113
Val Phe Gly Ser Thr Gly Asp Tyr Ser Asn Met Cys Asn Ser Asp Val
        260                 265                 270

AGT GTC GAC GCT ATG ATC CAC AAA ACG TAT ATA GAT GTC AAT GAA GAG    1161
Ser Val Asp Ala Met Ile His Lys Thr Tyr Ile Asp Val Asn Glu Glu
    275                 280                 285

TAT ACA GAA GCA GCT GCA GCA ACT TGT GCG CTG GTG GCA GAC TGT GCA    1209
Tyr Thr Glu Ala Ala Ala Ala Thr Cys Ala Leu Val Ala Asp Cys Ala
290                 295                 300                 305

TCA ACA GTT ACA AAT GAG TTC TGT GCA GAT CAT CCG TTC ATC TAT GTG    1257
Ser Thr Val Thr Asn Glu Phe Cys Ala Asp His Pro Phe Ile Tyr Val
                310                 315                 320

ATT AGG CAT GTC GAT GGC AAA ATT CTT TTC GTT GGT AGA TAT TGC TCT    1305
Ile Arg His Val Asp Gly Lys Ile Leu Phe Val Gly Arg Tyr Cys Ser
            325                 330                 335

CCA ACA ACT AAT TAAATCACAT TCTTAATATT AGAATATTAG AATATTATAT        1357
Pro Thr Thr Asn
        340

AGTTAAGATT TTTACTAATT GGTTAACCAT TTTTTTAAAA AAATAGAAAA AAAACATGTT  1417

ATATTAGCGA GGGTCGTTAT TCTTCCAATT GCAATTGGTA AGATGACGGC C           1468
```

FIG. 12B

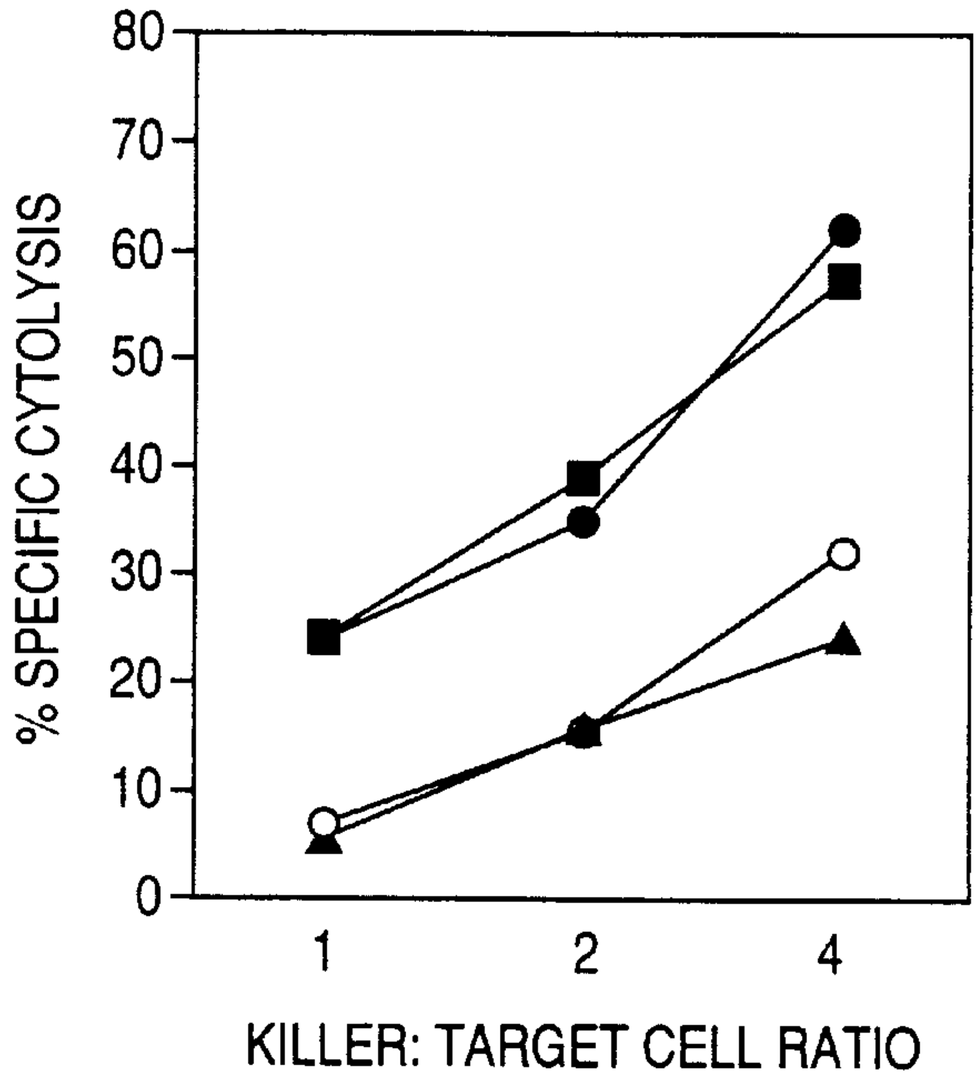


FIG. 13A

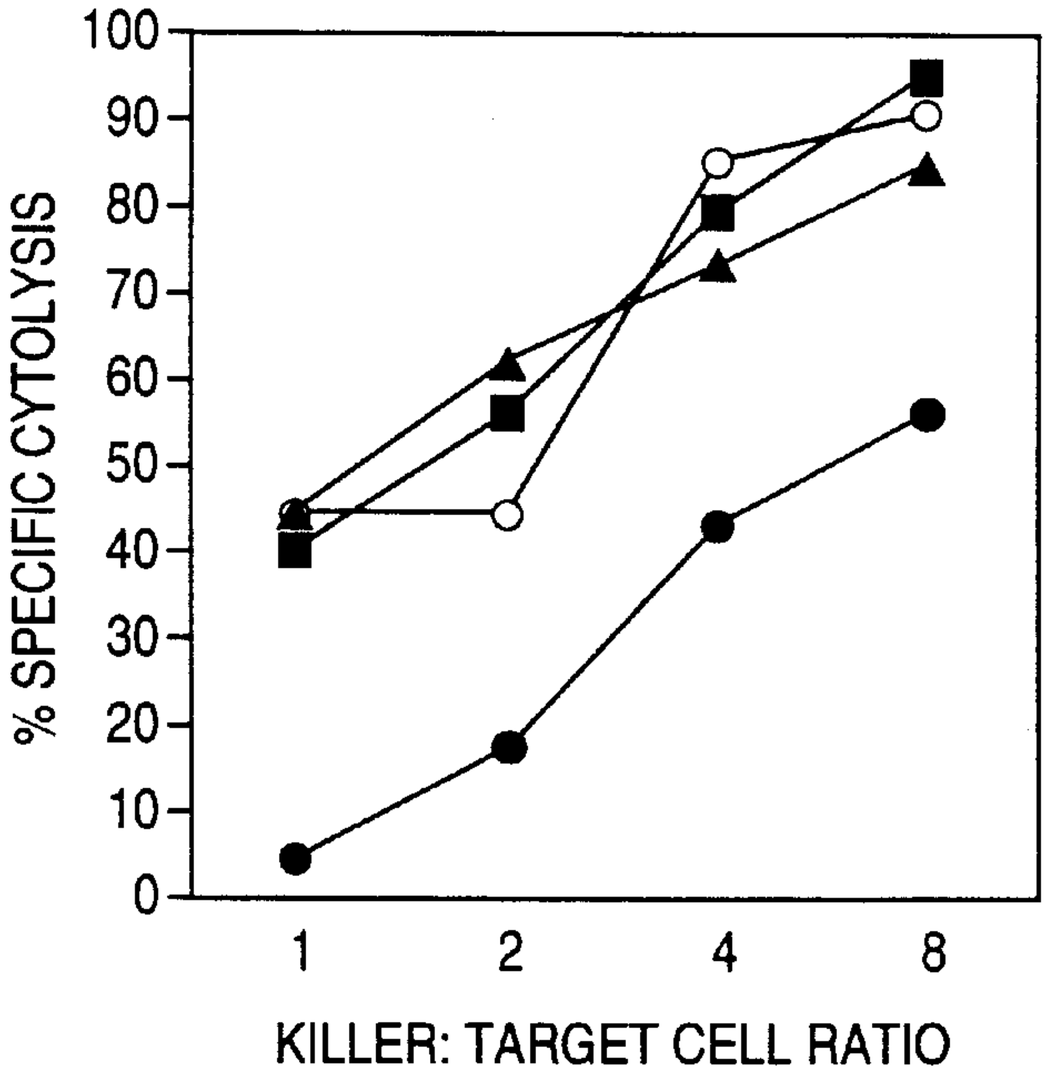


FIG. 13B

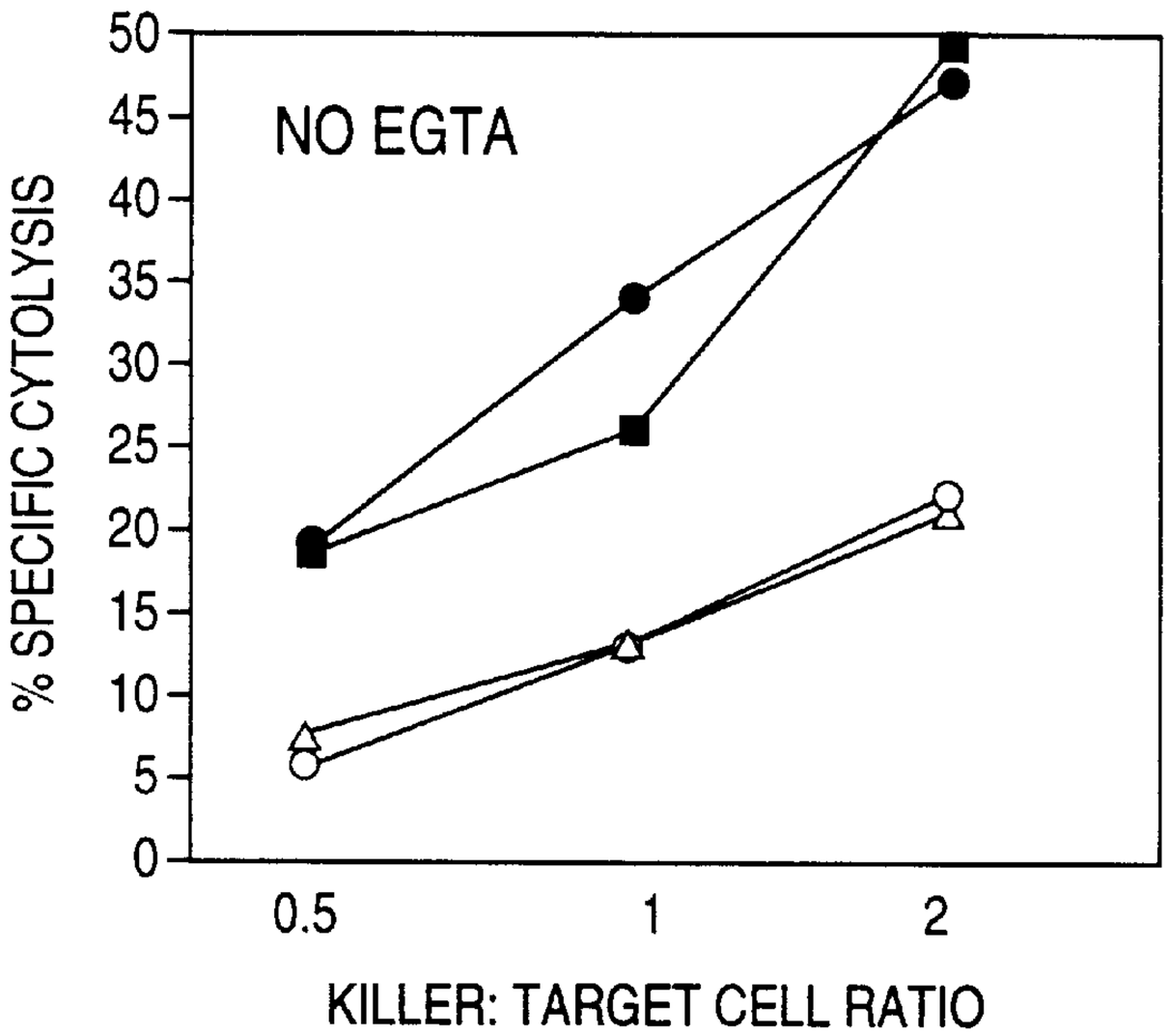


FIG. 14A

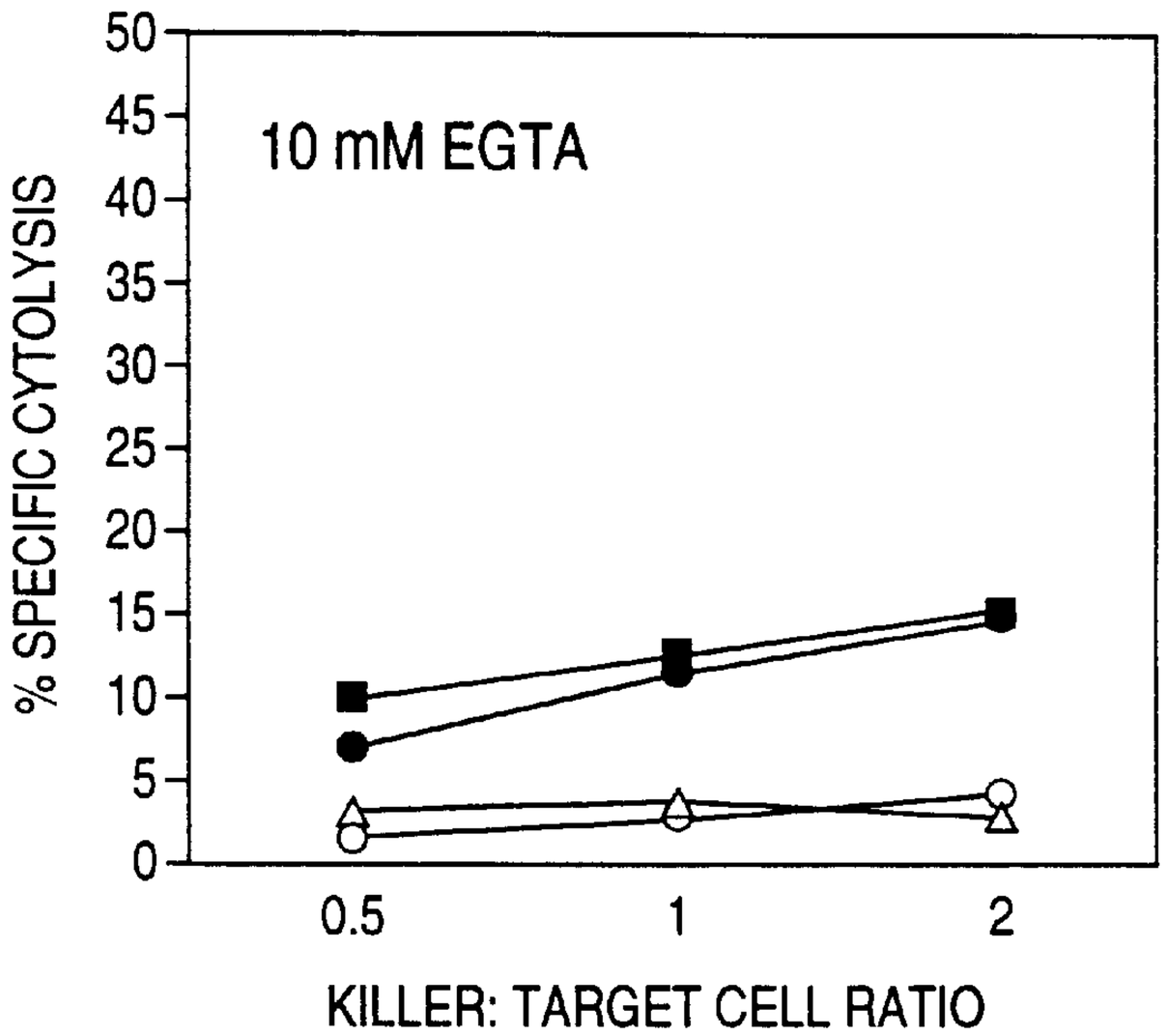


FIG. 14B

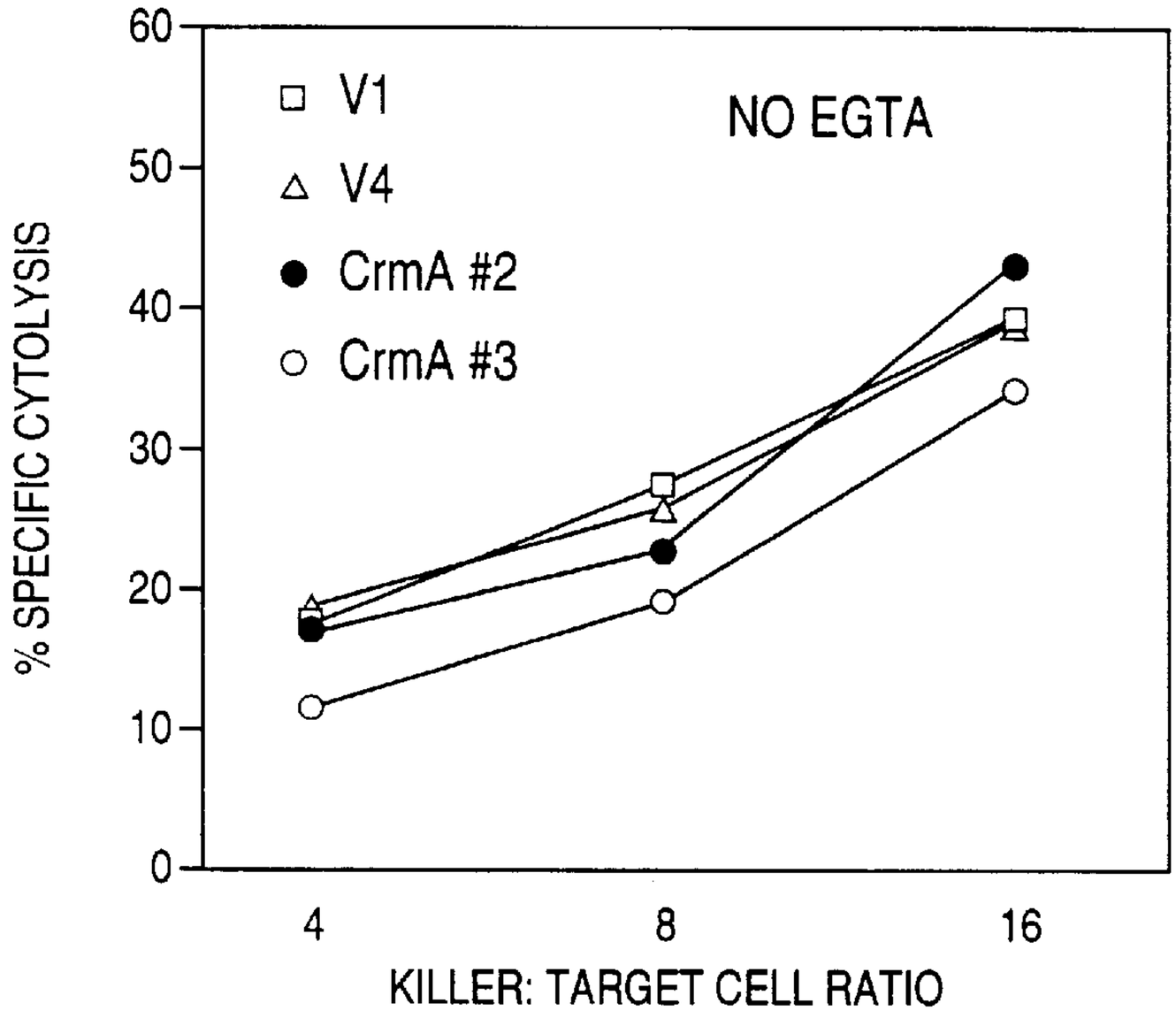


FIG. 15A

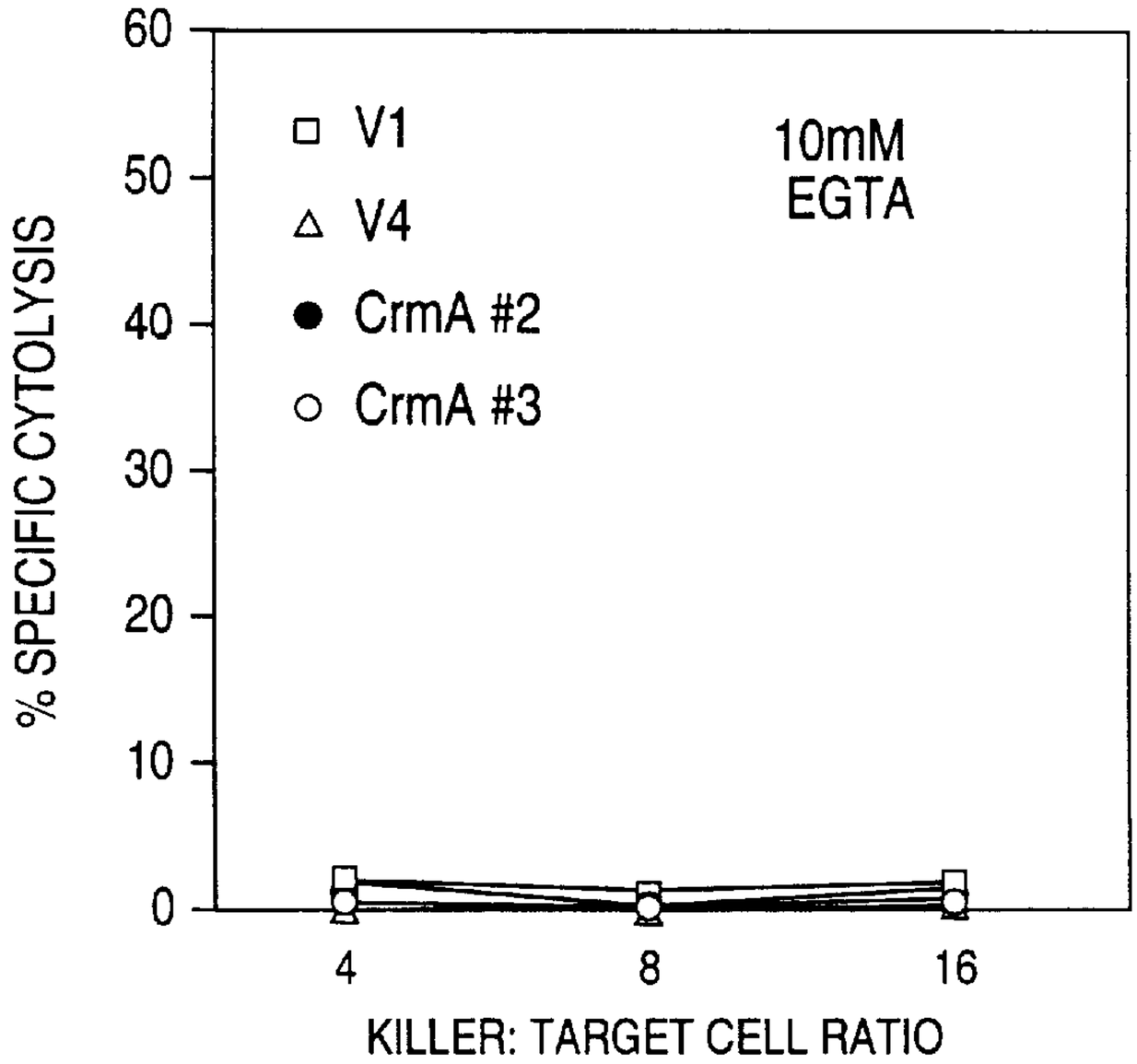


FIG. 15B

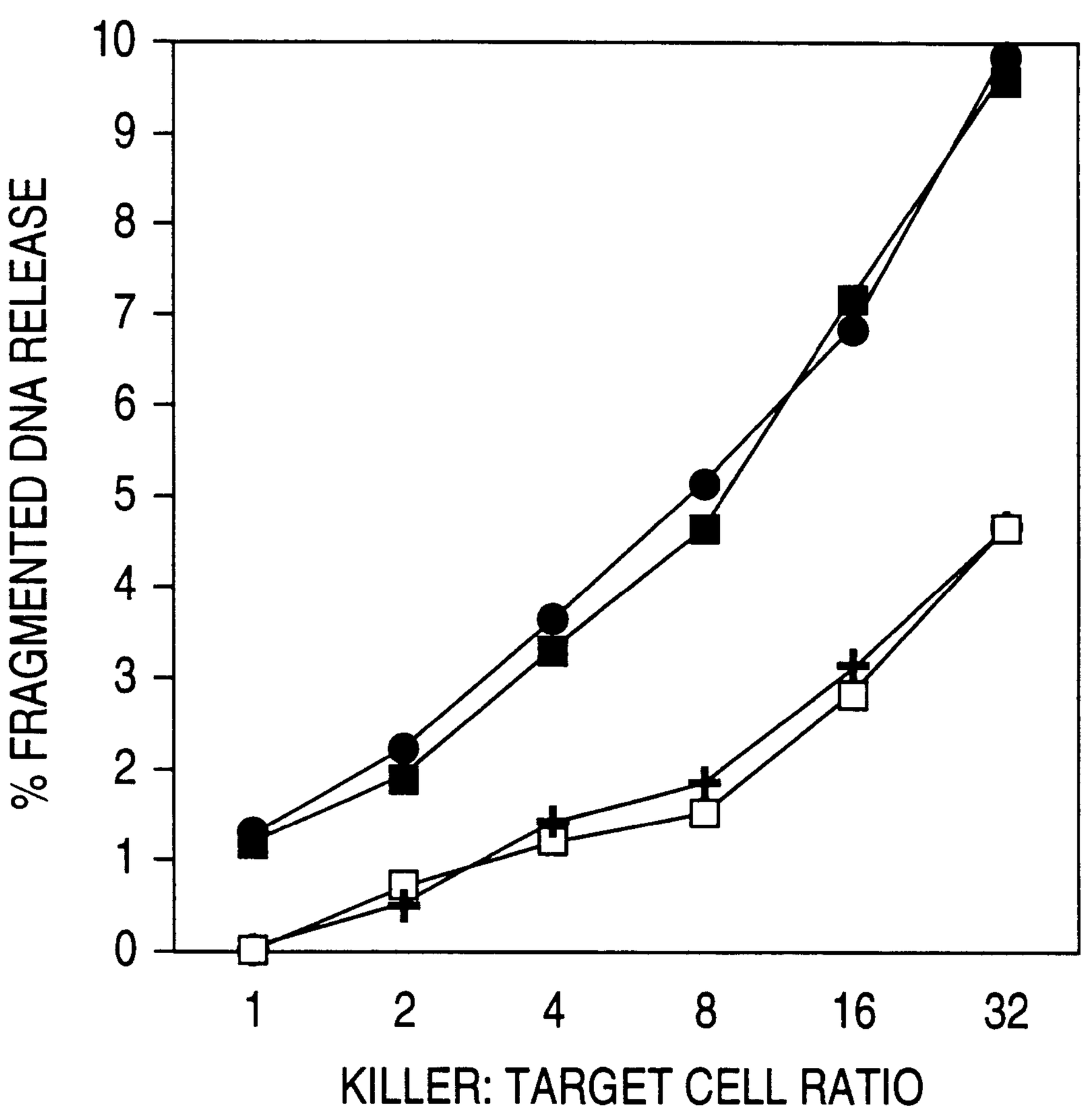

% FRAGMENTED DNA RELEASE
0
1
2
3
4
5
6
7
8
9
10
1
2
4
8
16
32
KILLER: TARGET CELL RATIO

FIG. 16

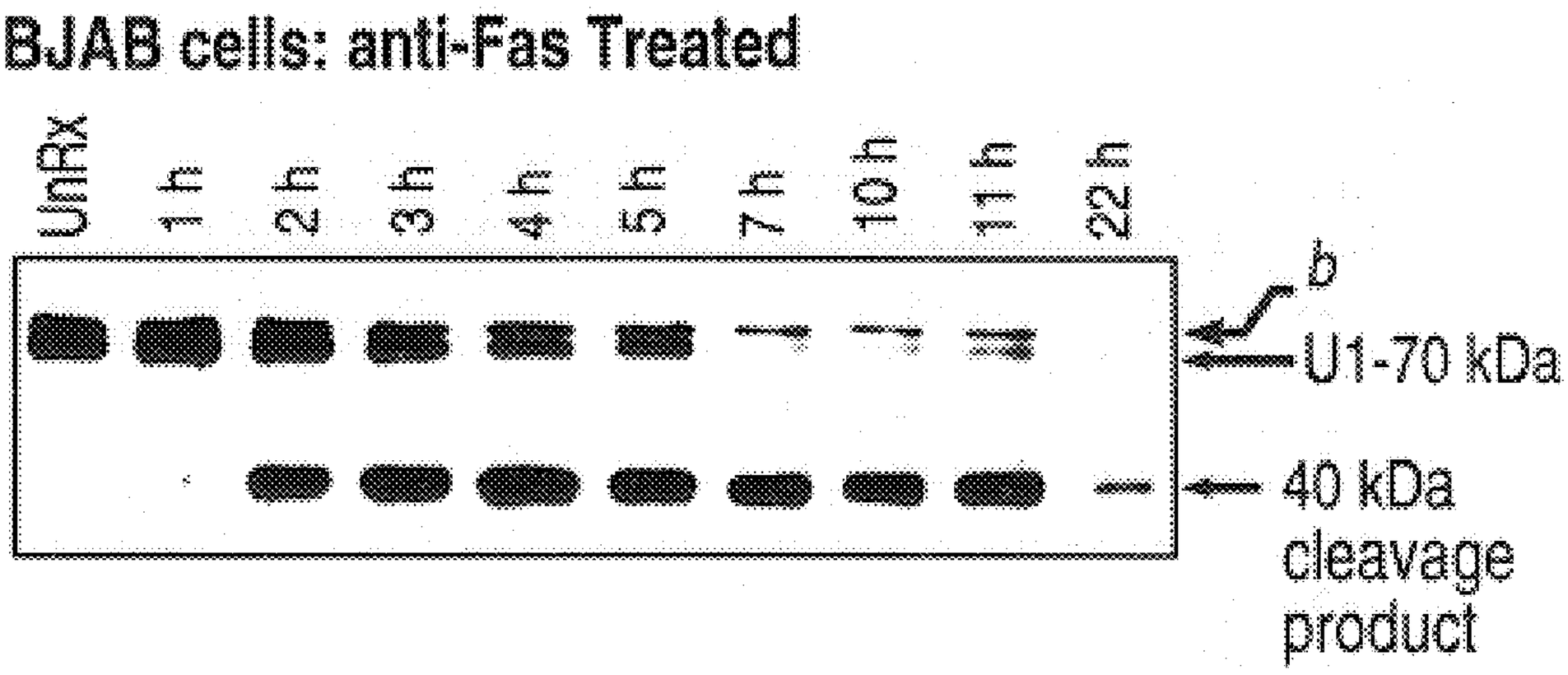


FIG. 17A

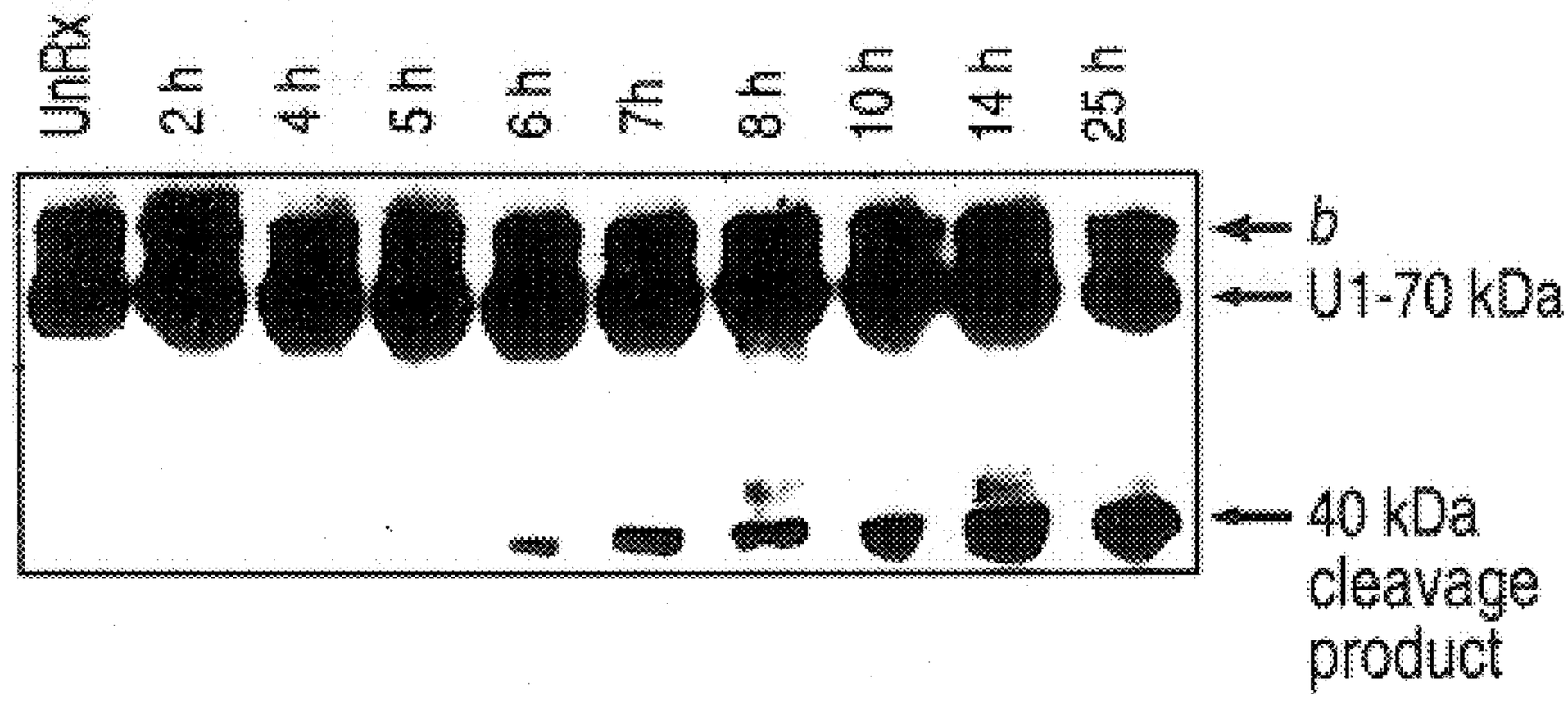


FIG. 17B

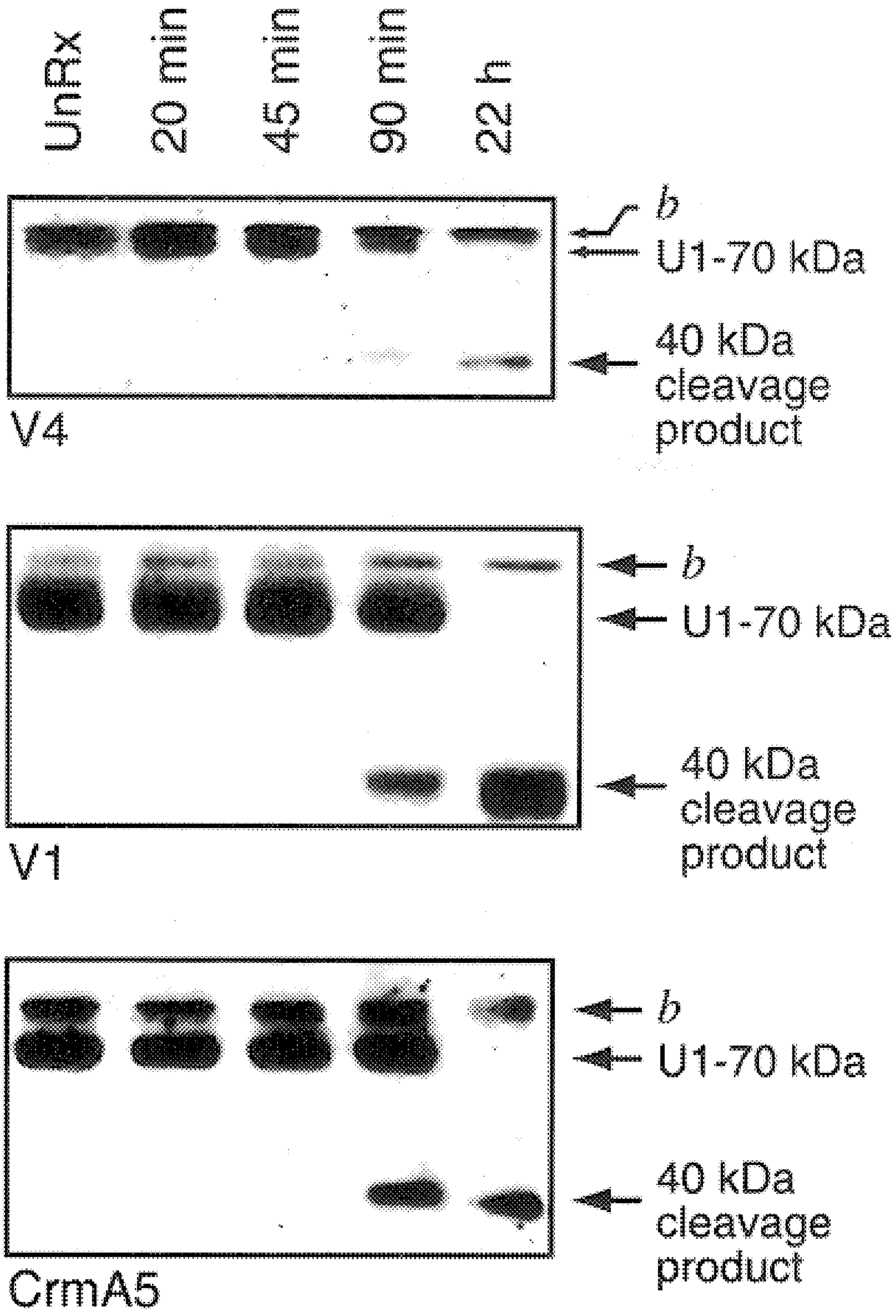

UnRx
20 min
45 min
90 min
22 h
b
U1-70 kDa
40 kDa cleavage product
V4
b
U1-70 kDa
40 kDa cleavage product
V1
b
U1-70 kDa
40 kDa cleavage product
CrmA5

FIG. 18A

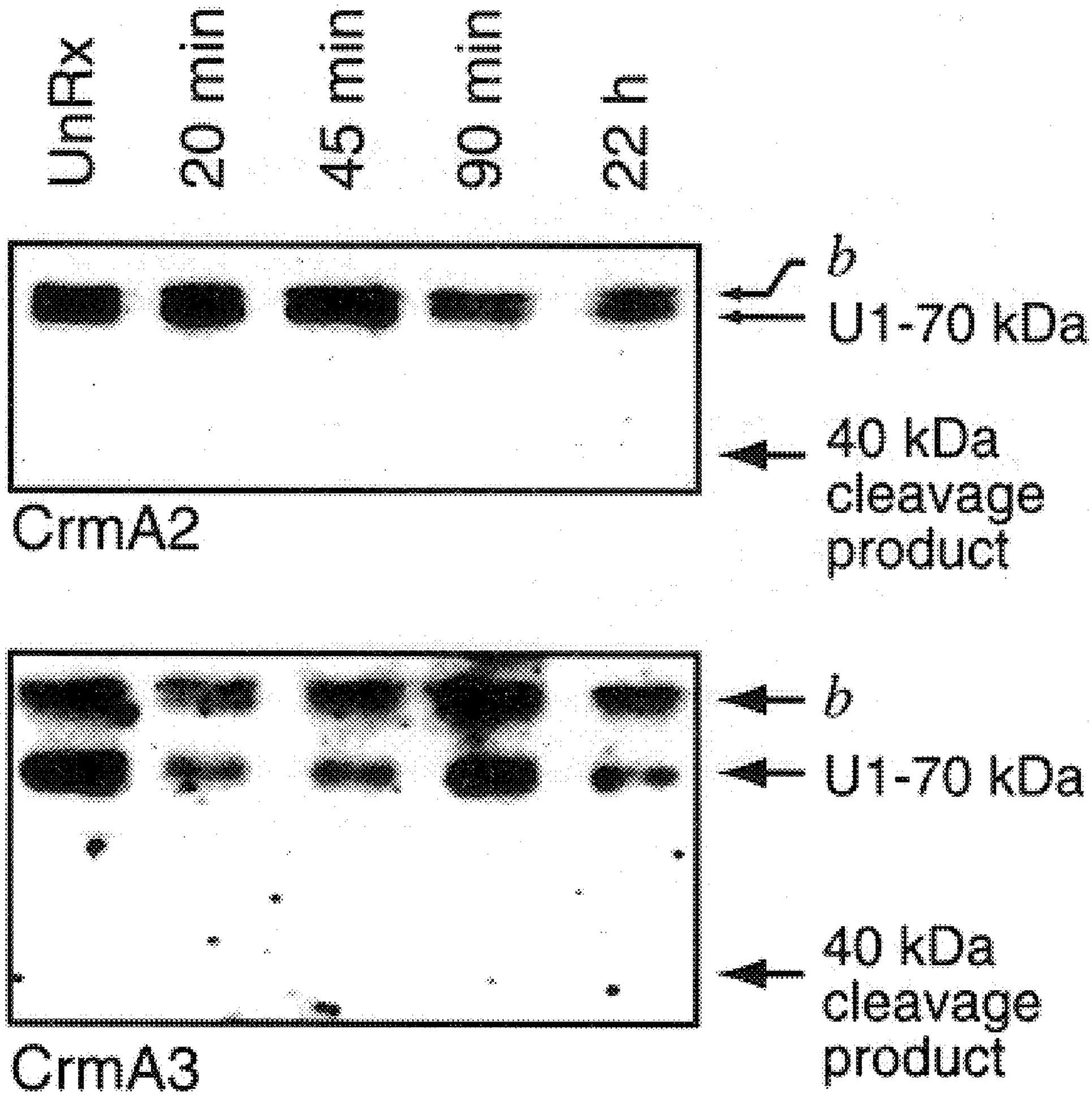

RX
5'
20
45
60
22
b
U1-70 kDa
40 kDa cleavage product
CrmA2
b
U1-70 kDa
40 kDa cleavage product
CrmA3

FIG. 18B

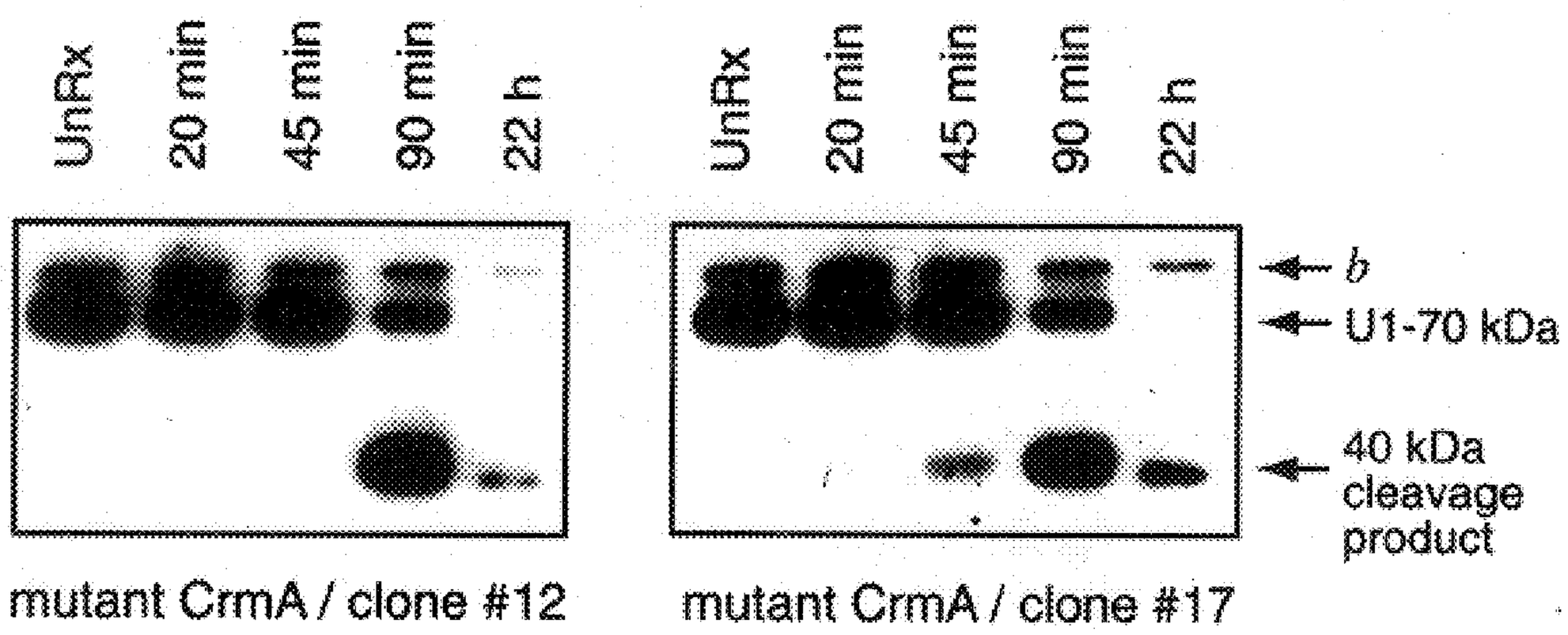


FIG. 18C

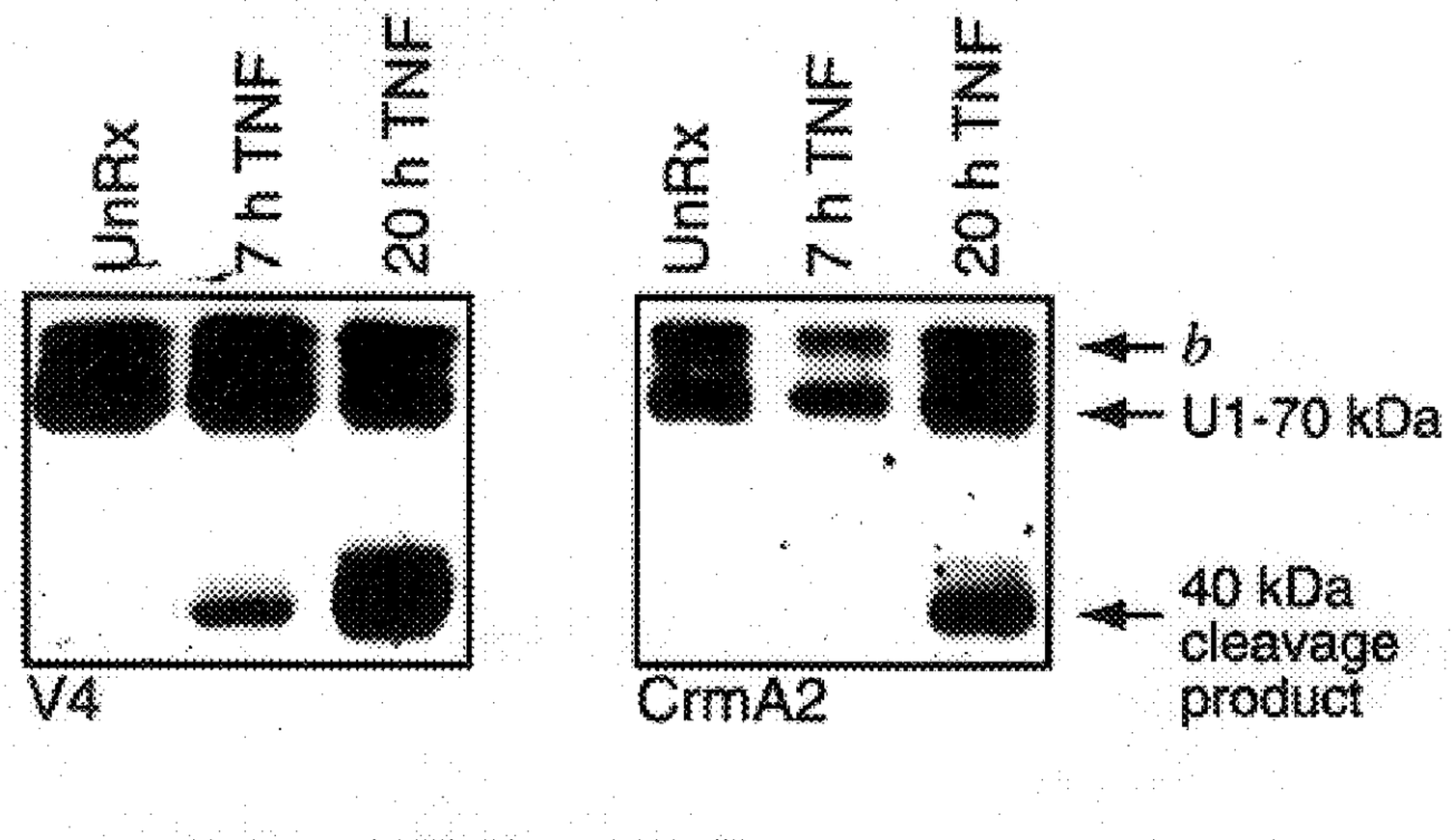


FIG. 19A

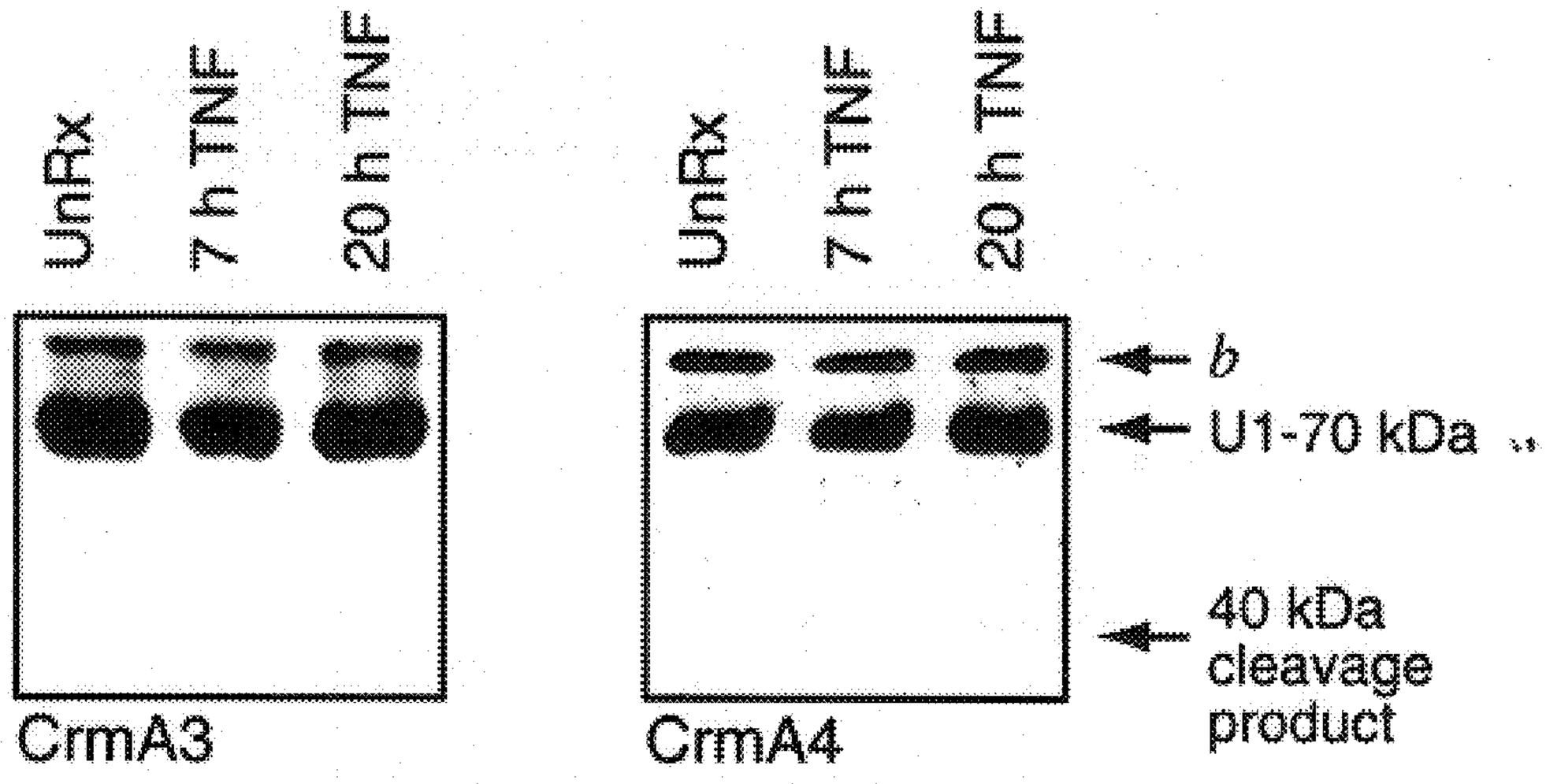


FIG. 19B

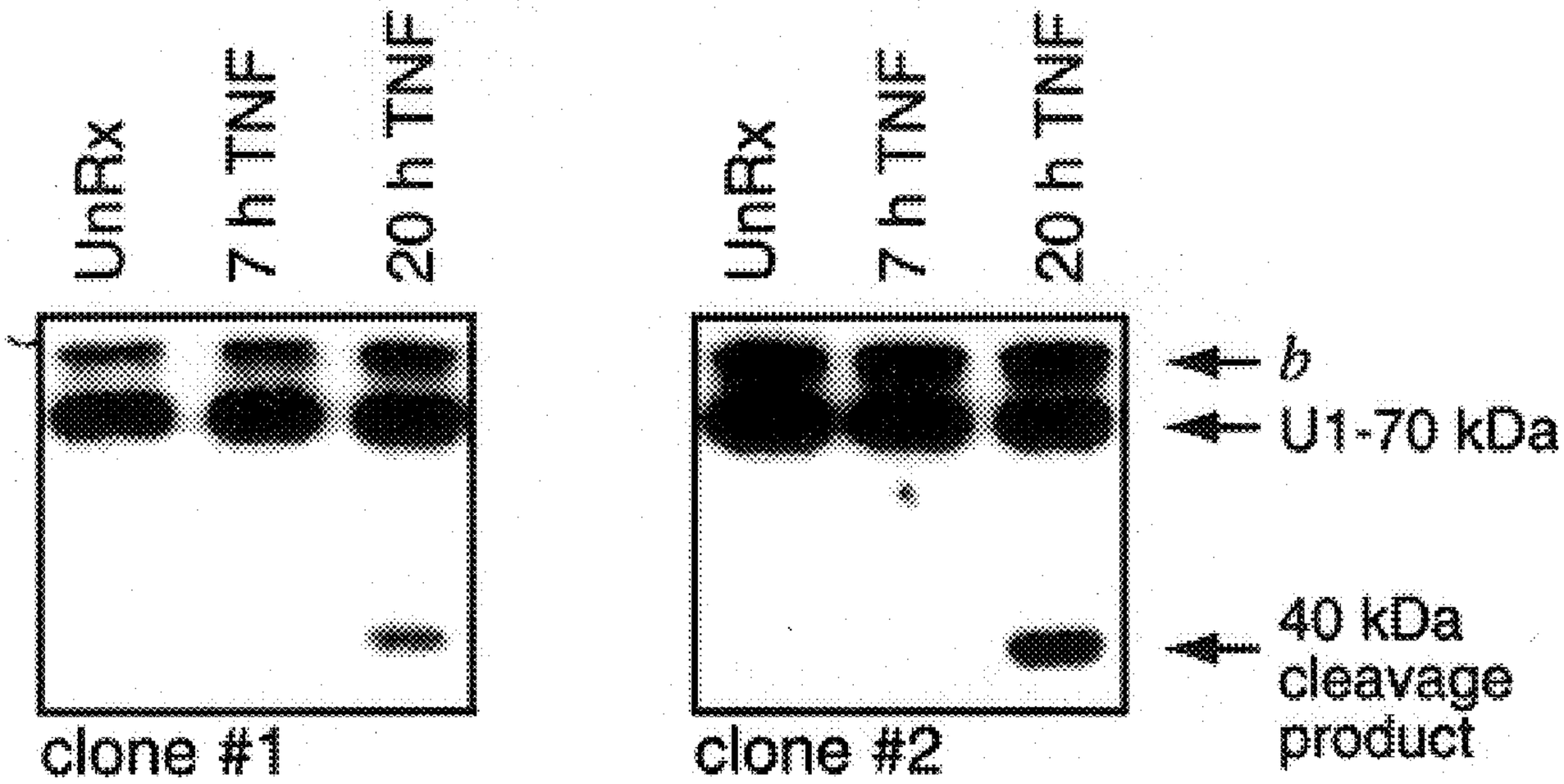
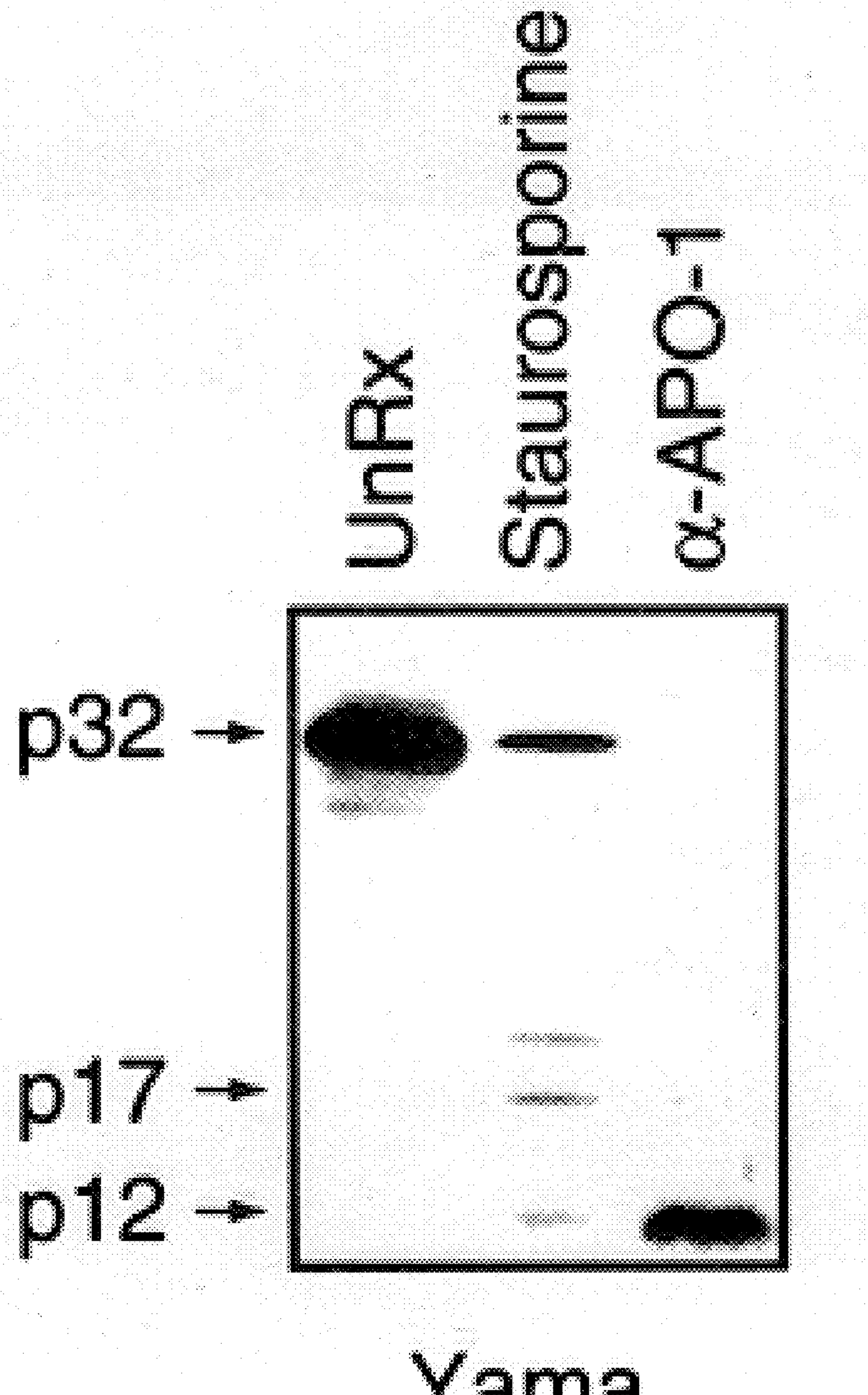


FIG. 19C



Yama

FIG. 20

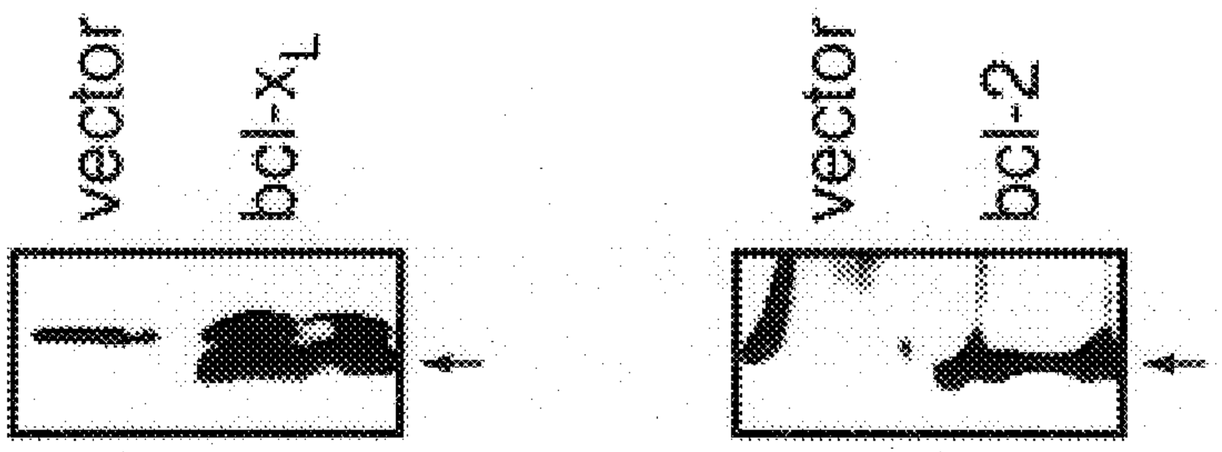


FIG. 21A

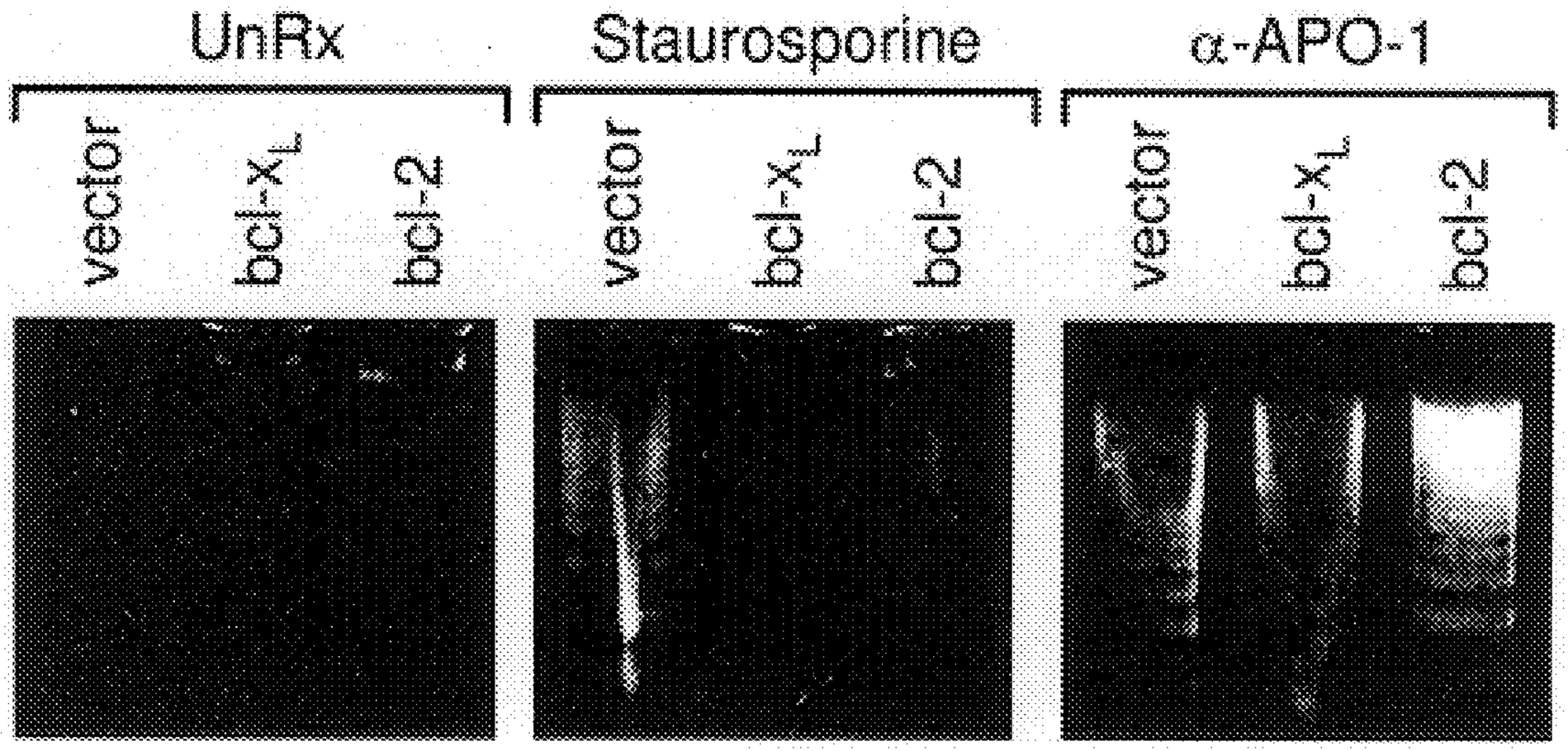


FIG. 21B

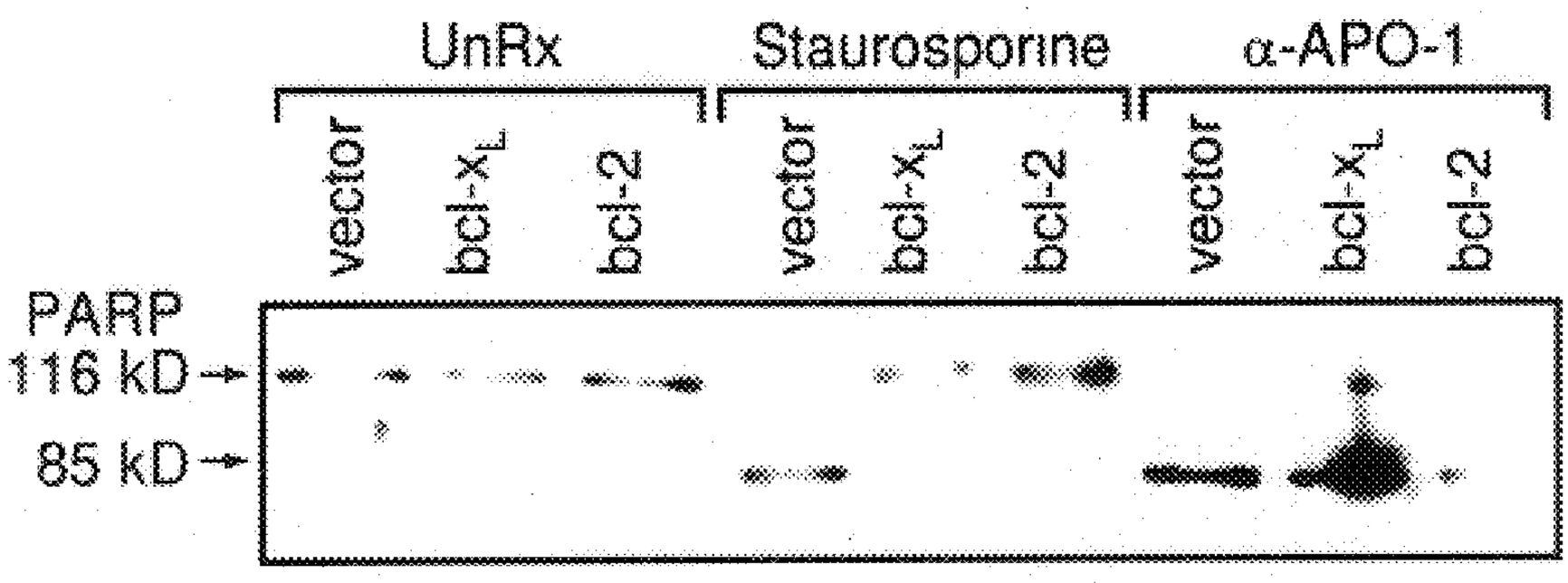


FIG. 21C

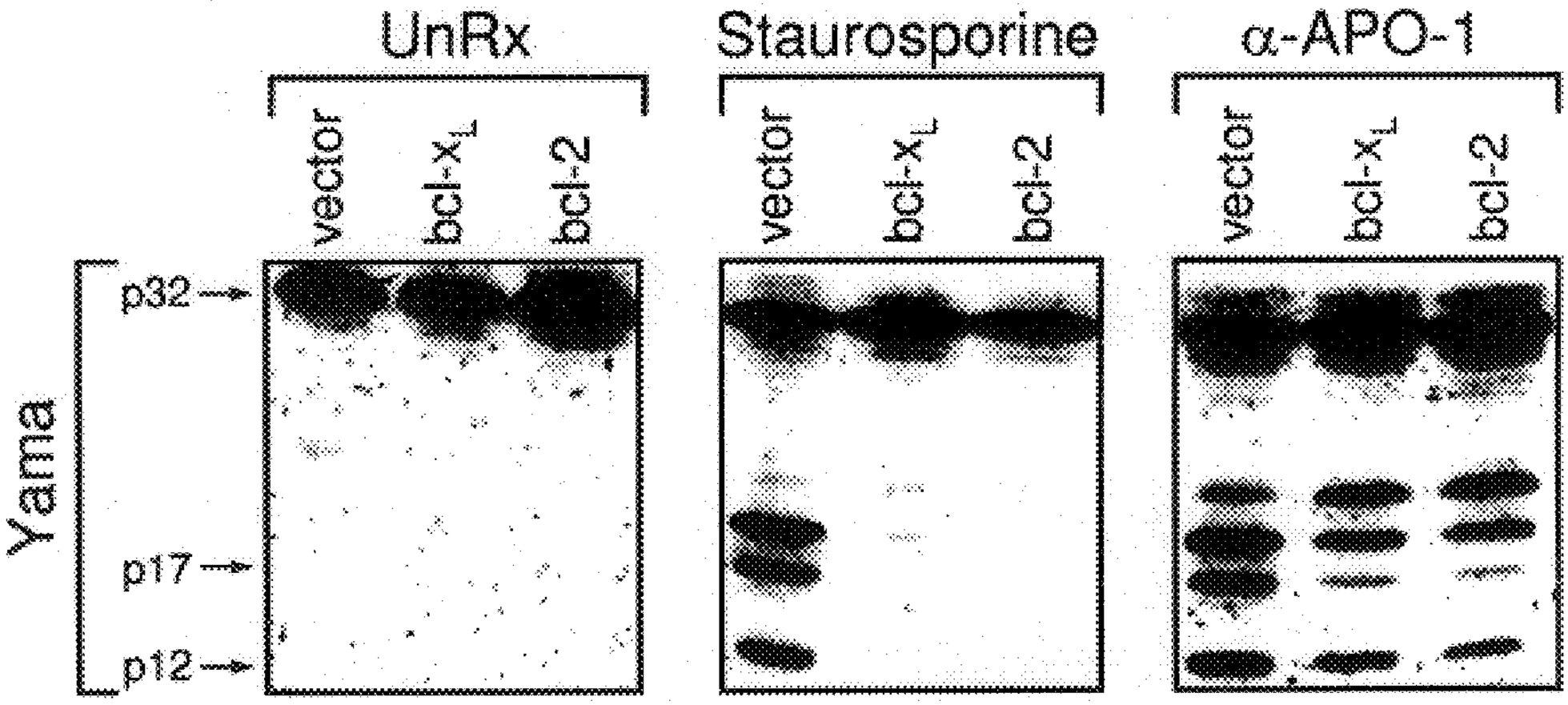


FIG. 21D

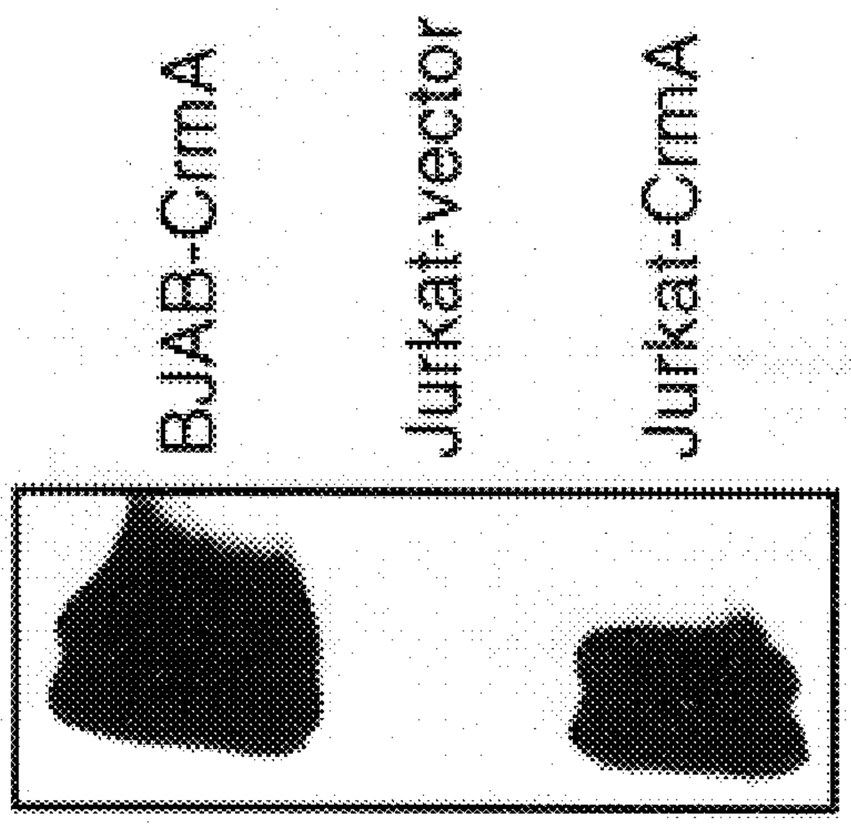


FIG. 22A

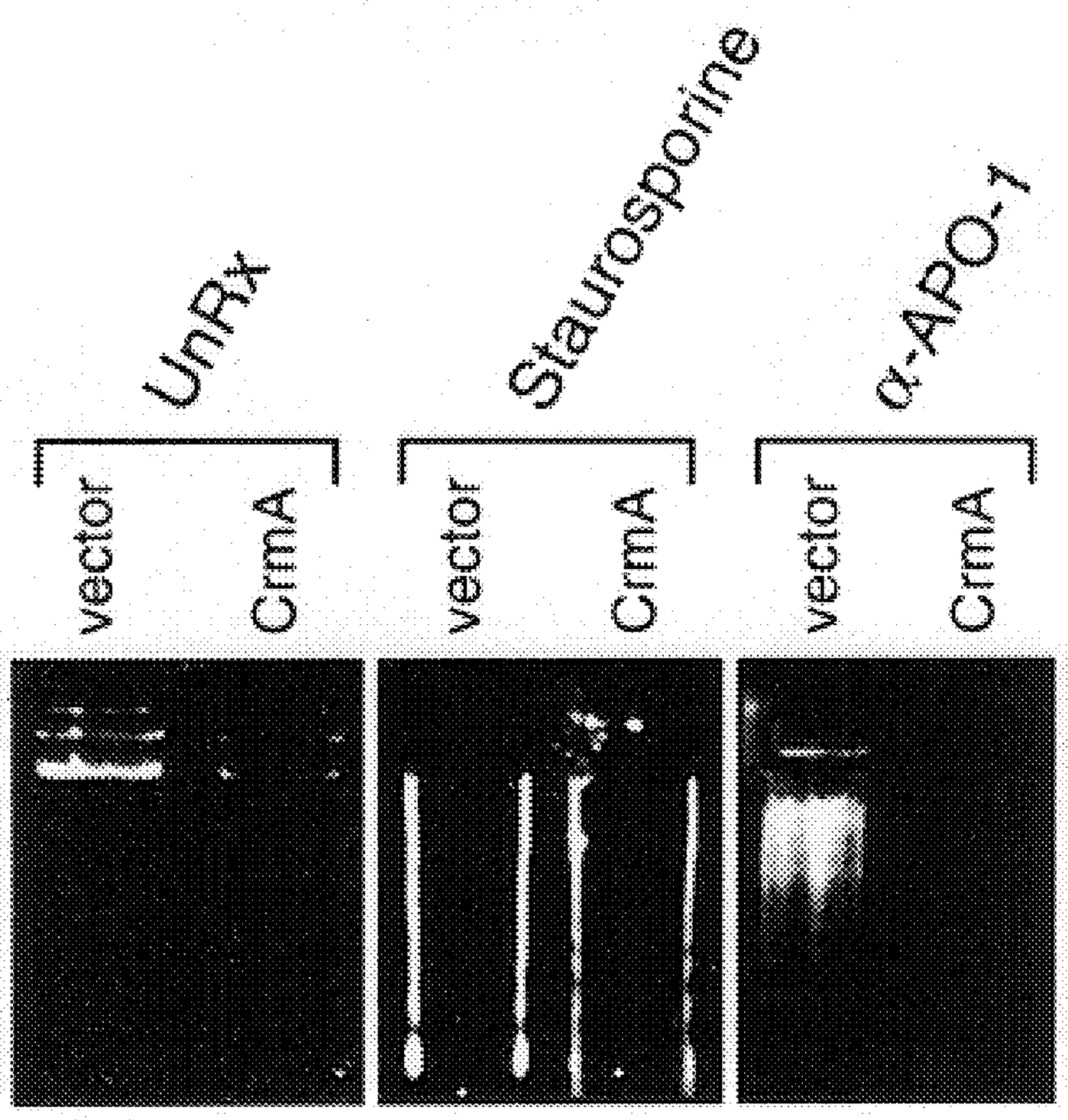


FIG. 22B

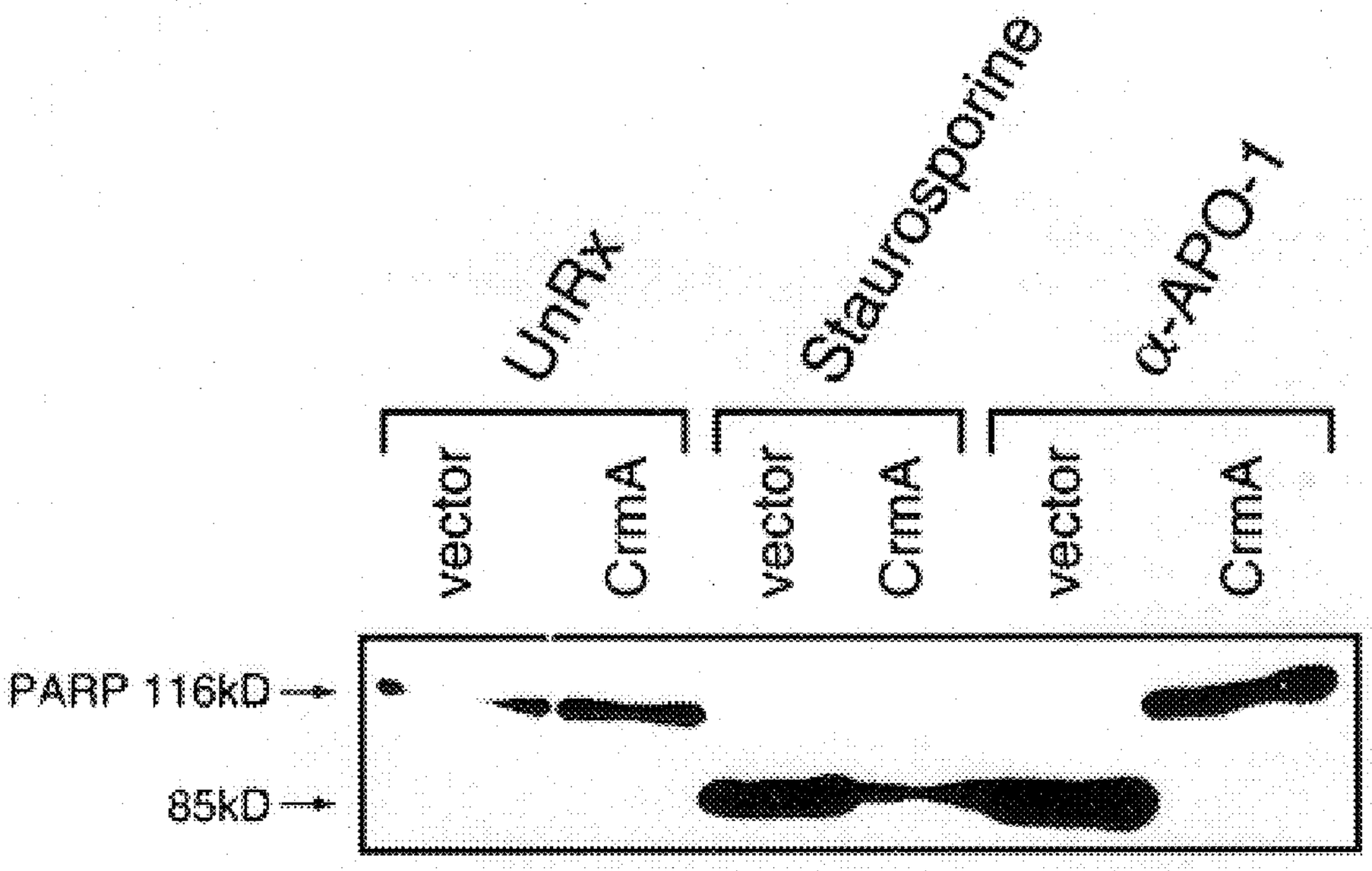


FIG. 22C

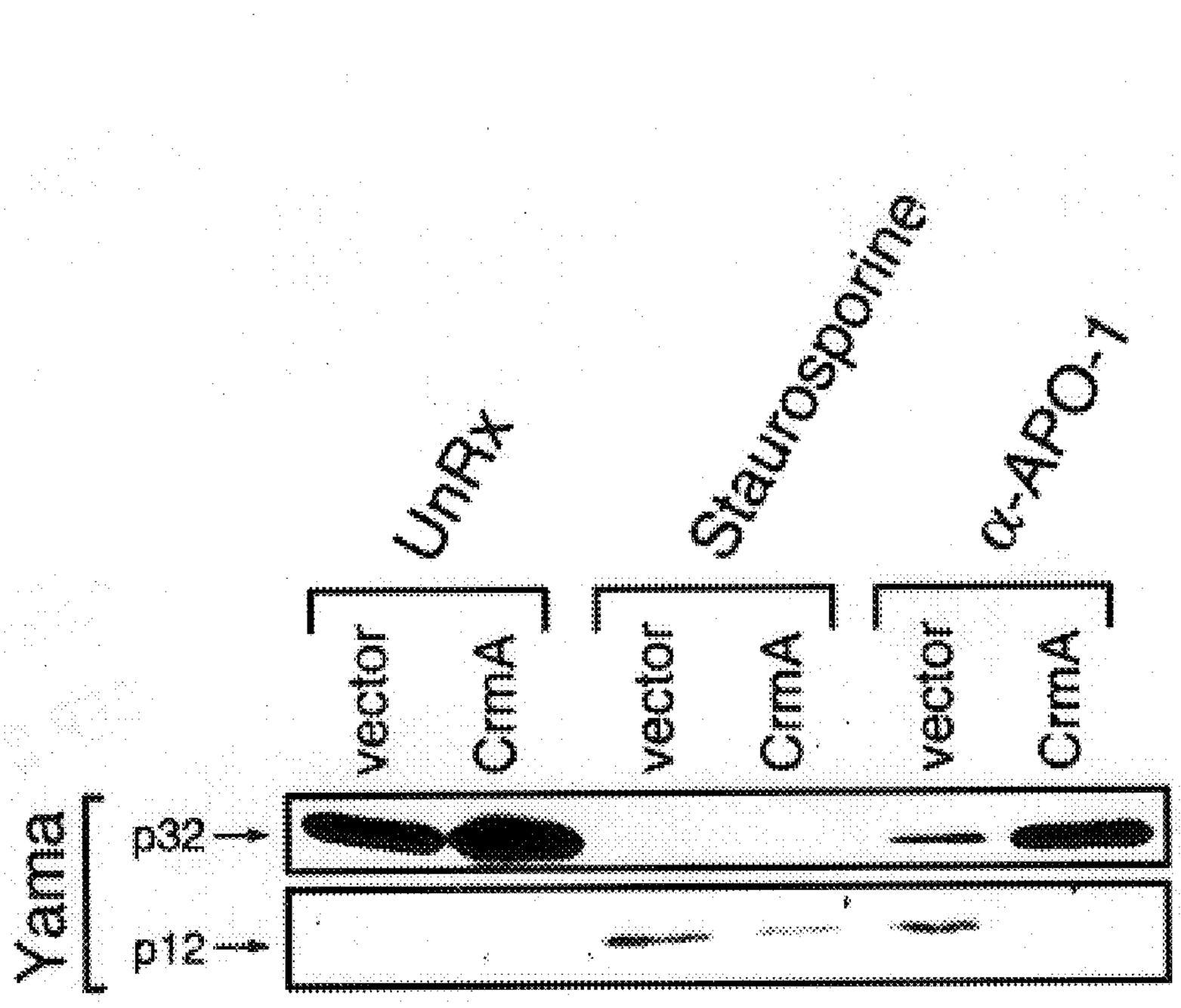


FIG. 22D

METHOD AND COMPOSITION FOR REGULATING APOPTOSIS

This application is a continuation-in-part of U.S. Ser. No. 08/389,812, and 08/457,731, filed Feb. 13, 1995 and June 1, 1995, respectively, the contents of which are hereby incorporated by reference into the present disclosure.

This invention was made in part with support from the United States government under Grant Nos. CA64803 and CA61348, each from the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

TECHNICAL FIELD

This invention relates to a protease that is an effector component of a mammalian cell death pathway. More specifically, it relates to a nucleic acid molecule encoding the protease, the recombinantly produced protease, purified protease and use of the protease. It also relates to methods for regulating apoptosis in a population of cells.

BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death (PCD), is of fundamental importance to biological processes including embryogenesis, maintenance of tissue homeostasis, normal cellular development of multicellular organisms, elimination of virus-infected cells, and the development of the immune system (Ellis et al. (1991) *Ann. Rev. Cell Biol.* 7:663–698). It is a type of cell death that is fundamentally distinct from degenerative death or necrosis in that it is an active process of gene-directed cellular self-destruction which in some instances, serves a biologically meaningful homeostatic function. Necrosis, in contrast, is cell death occurring as the result of severe injurious changes in the environment of infected cells. For a general review of apoptosis, see Tomei, L. D. and Cope, F. O. *Apoptosis: The Molecular Basis of Cell Death* (1991) Cold Spring Harbor Press, N.Y.; Tomei, L. D. and Cope, F. O. *Apoptosis II: The Molecular Basis of Apoptosis in Disease* (1994) Cold Spring Harbor Press, N.Y.; and Duvall and Wyllie (1986) *Immun. Today* 7(4) :115–119.

Morphologically, apoptosis is characterized by the rapid condensation of the cell with preservation of membranes. Synchronistically with the compaction of chromatin, several biochemical changes occur in the cell. Nuclear DNA is cleaved at the linker regions between nucleosomes to produce fragments that are easily demonstrated by agarose gel electrophoresis wherein a characteristic ladder develops.

Numerous triggers for apoptosis have been identified that induce cell death. The Fas antigen (CD95/APO-1) is a member of the tumor necrosis factor (TNF) receptor superfamily. It is a transmembrane protein with wide tissue distribution. Fas triggers apoptosis upon activation when bound to its ligand or to agonistic anti-Fas antibodies. Cytotoxic T-lymphocytes activate Fas on target cells, inducing cytolysis. TNF also is known to induce apoptosis when cross-linked by its ligand or an agonist antibody.

In addition to being linked to the many biological processes identified above, apoptosis also occurs as a result of human immunodeficiency virus (HIV) infection of $CD4^+$ T lymphocytes (T cells). Indeed, one of the major characteristics of AIDS is the gradual depletion of $CD4^+$ T lymphocytes during the development of the disease. Several mechanisms, including apoptosis, have been suggested to be responsible for the CD4 depletion. The depletion of $CD4^+$ T cells results in the impairment of the cellular immune

response. It has been proposed that an inappropriate activation-induced T cell PCD causes the functional and numerical abnormalities of T_H cells from HIV-infected patients, that leads to the near collapse of the patient's immune system. The mechanism by which the Fas antigen initiates the apoptotic cascade, and its various components, remains to be fully elucidated. However, this invention has identified several components of the apoptotic cascade and methods to modulate apoptosis.

SUMMARY OF THE INVENTION

This invention provides non-naturally occurring and isolated, naturally occurring nucleic acid molecules (polynucleotides) which encode proteins designated Yama, Pro-Yama, activated Yama, p20 Yama, p 11 Yama and mutant Yama.

This invention also provides a recombinant nucleic acid molecule having a nucleic acid molecule sequence as shown in FIG. **1**. (SEQ. ID. NO: 1) Further provided by this invention are fragments of the above-mentioned nucleic acid molecules. In one embodiment, the fragments have at least 8 nucleic acid bases for use as probes or primers. Specific examples of these fragments are nucleic acid molecules coding for the polypeptides designated herein as p20 Yama and p11 Yama. Also provided are the complementary DNA and RNA polynucletoides to these nucleic acid molecules.

Also provided by this invention is a recombinant nucleic acid molecule encoding a polypeptide having the amino acid sequence depicted in FIG. **1** or a fragment of this amino acid sequence or polypeptide. Further provided by this invention is a non-naturally occurring nucleic acid molecule encoding mutant CrmA protein and a dominant inhibitory Yama. Vectors and host cells containing these nucleic acid molecules are further provided.

Purified and recombinantly produced proteins and polypeptides designated Pro-Yama, activated Yama, p20 Yama, p 11 Yama, mutant CrmA and mutant Yama also are provided herein.

This invention also provides a non-naturally occurring nucleic acid molecule encoding mutant CrmA protein and nucleic acid molecules (RNA and DNA) complementary to this molecule and the polypeptides encoded by them.

These nucleic acid molecules can be inserted into vectors and such vectors are further provided by this invention. Also provided herein are host cells, i.e., procaryotic and eucaryotic cells, containing these nucleic acid molecules that have been inserted therein.

In one embodiment, the Yama protein of this invention is a substantially purified naturally occurring zymogen polypeptide having an apparent molecular weight of about 32 kDa as determined by SDS-PAGE which upon activation. promotes apoptosis. In a separate embodiment, it is a non-naturally occurring zymogen polypeptide having an apparent molecular-weight of about 32 kDa as determined by SDS-PAGE which upon activation promotes apoptosis. In a still further embodiment, it is a non-naturally occurring polypeptide having an amino acid sequence comprising the sequence depicted in FIG. **1**. In a still further embodiment, it is a non-naturally occurring polypeptide comprising a fragment having an apparent molecular weight of about 20 kDa or 11 kDa as determined by SDS-PAGE. Actual molecular weights of these polypeptides are 17 kDa and 12 kDa, respectively.

This invention also provides a complex comprising CrmA polypeptide and a protein designated Yama.

Process for producing a Yama protein or polypeptide also is provided herein. This process requires a host cell, wherein

the host cell contains a nucleic acid molecule encoding the Yama protein and wherein the nucleic acid molecule is operatively linked to a promoter of RNA transcription, growing the host cell under suitable conditions such that the nucleic acid is transcribed and translated into the Yama protein or polypeptide protein. An alternative process of this invention provides chemically synthesizing a Yama protein or polypeptide by chemically linking the amino acids according to the amino acid sequence in an orientation and under suitable conditions so as to produce the Yama protein or polypeptide. Further provided herein is a process for chemically replicating a nucleic acid molecule encoding a Yama protein or polypeptide by chemically linking the nucleotides according to the nucleic acid sequence in an orientation and under suitable conditions so as to produce the nucleic acid.

Methods of modulating a cellular function regulated by the Fas receptor pathway in a suitable cell also is provided by this invention. In one embodiment, the method requires introducing into the cell a Fas-regulating nucleic acid molecule and growing the cell under suitable conditions such that the nucleic acid is transcribed and translated into Fas-regulating protein in the cell. Two examples of these proteins are Yama nucleic acid and a nucleic acid having CrmA biological activity. The introduction into the cell can be effected in vitro, in vivo or ex vivo.

Also provided herein is a method for preventing or inhibiting apoptosis in a suitable cell by introducing into the cell an effective amount of a nucleic acid molecule coding for a gene product having CrmA biological activity and under suitable conditions such that apoptosis is prevented or inhibited. Examples of this nucleic acid molecules are cowpox virus CrmA DNA or a biologically active fragment thereof. Alternatively, the method can be practice using a nucleic acid molecule coding for a dominant inhibitory Yama.

A method for maintaining T cell viability in a subject infected with the human immunodeficiency virus is further provided herein by administering to the subject an effective amount of a nucleic acid molecule coding for a gene product having CrmA biological activity and under suitable conditions such that T cells remain viable. This method also can be practiced using a nucleic acid molecule comprises a polynucleotide coding for a dominant inhibitory Yama.

Antibodies that that specifically recognize and binds a Yama protein or a fragment thereof also are provided herein. The antibodies can be polyclonal or monoclonal. This invention further provides fragments of these antibodies as well as a recombinant or chimeric molecule comprising the antibody or antibody fragment. Anti-idiotypic antibodies of these antibodies also are provided herein as well as the hybridoma cell lines producing the claimed monoclonal antibodies. Compositions containing these antibodies also are provided.

A method for identifying agents that are involved in the apoptotic pathway in a suitable cell is provided herein. The method requires the steps of a) providing cell cultures or tissue culture having a cell surface receptor that mediates apoptosis; b) exposing the cell cultures or tissue cultures to preliminary conditions necessary for apoptosis; c) dividing the cell cultures or tissue cultures into a test sample and a control sample; d) contacting the agent to be tested with the cell culture or tissue culture of the test sample; e) contacting a nucleic acid molecule coding for a gene product having CrmA biological activity or CrmA gene product to the control sample under conditions to inhibit apoptosis with the cell culture or tissue culture of the control sample; f) contacting the test sample with a nucleic acid molecule coding for a gene product having CrmA biological activity or CrmA gene product to the test sample under conditions to inhibit apoptosis of the cells; g) culturing the samples of steps e) and f) under conditions to inhibit apoptosis; and h) assaying the samples of step g) for apoptosis. In one embodiment of this method, the additional step of identifying an agent that augments apoptosis and is inhibitable by CrmA gene product, by the absence of apoptotic morphological changes or the absence or inhibition of cell death of the test sample. Assaying for the cleavage of PARP or U1-70 kDA proteins is one means to test for apoptotic activity and whether the agent will modify the apoptotic cascade.

This invention also provides a method for screening for candidate agents having a biological function in the apoptotic pathway. The method requires a) incubating an effective amount of Pro-Yama with an activating agent under suitable conditions to activate Yama; b) dividing activated Yama into at least two reaction solutions; c) contacting activated Yama with an effective amount of the agent to be tested; d) contacting a separate activated Yama with an effective amount of CrmA under suitable conditions such that is Yama is inhibited e) contacting an effective amount of PARP to the solutions of step c) and step d); and f) analyzing the solutions of step e), to determine if the agent inhibited cleavage of PARP to its 85 kDa form, the absence of the 85 kDa form being an indication that the agent is candidate having a biological function in the apoptotic pathway. This method also can be practice by incubating with the U1-70 kDa protein described herein and assaying to determine if the agent inhibited cleavage of the protein to the 40 kDa form. Agents identified by this method are within the scope of this invention.

This invention further provides compositions and methods for preventing or inhibiting apoptosis in a suitable cell by introducing into the cell a nucleic acid molecule coding for a gene product or the gene product having CrmA biological activity such that induced apoptosis is prevented or inhibited. Suitable nucleic acid molecules for this method indicate, but are not limited to, dominant inhibitory Yama or the CrmA gene product.

Further provided by this invention are compositions and methods for maintaining T cell viability in a subject infected with or susceptible to infection with the human immunodeficiency virus by administering to the subject an effective amount of a nucleic acid molecule coding for a gene product having apototic biological activity or the gene product. These may be, for example, CrmA nucleic acid, a dominant inhibitory Yama nucleic acid, or the gene products of these nucleic acid molecules.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** (SEQ ID NO: 1 and SEQ ID NO: 2) shows the open reading frame and deduced amino acid sequence of the protein designated herein as Pro-Yama. In the nucleotide sequence shown in FIG. **1**, the initiator methionine begins at nucleotide 224.

FIGS. **2**A and **2**B show that Pro-Yama is a protease zymogen that, upon activation, cleaves PARP in vitro to the 85 kDa apoptotic fragment.

In the top panel of FIG. **2**A, it is shown that 6xHis-tagged Yama was expressed and labeled using [^{35}S]-Met in an in vitro transcription/translation reaction and purified by affinity chromatography on sequential DEAE-sepharose and nickel chelate columns as described in the experimental

section below. In vitro reactions were assembled in which 0.586 μg of purified PARP was incubated for 2 hours at 37° C. with either buffer only (Lane 1), ICE (Lane 2), purified Pro-Yama (Lane 3), or purified Pro-Yama after activation with ICE (Lane 4). Following incubation, one fifth of each reaction was analyzed by SDS-PAGE and immunoblotting using monoclonal antibody C-2-10 directed against PARP. Whole cell lysates from BJAB cells undergoing anti-Fas-induced apoptosis (Lane 5) or from untreated BJAB cells (Lane 6) were run alongside the in vitro reaction samples. Shown in the bottom panel are the results when equal quantities of the in vitro reactions represented in Lanes 1–4 of FIG. **2**A were resolved by SDS-PAGE and the dried gel was subjected to Phosphorimager analysis to assess the state of the radio-labeled Yama protein. The filed-in arrow indicates the migration of purified Pro-Yama, which is designated as the full-length p32 form. The open arrows indicate the two major proteolytic fragments observed after activation of Pro-Yama by ICE, and are presumed to correspond to the putative p20 and p11 subunits predicted from cleavage at Asp residues in Pro-Yama.

FIG. **2**B shows that cleavage of PARP to an 85 kDa fragment is a characteristic feature of both Fas- and TNF-induced apoptosis. In the top panel, BJAB cells were either left untreated (UnRx) or treated with agonist anti-Fas antibody (250 ng/ml) for the indicated time periods and cell lysates prepared and analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10 as described in the experimental section below. In the bottom panel, MCF7 cells were either left untreated (UnRx) or treated with recombinant TNF (40 ng/ml) for the indicated time periods. Cell lysates were similarly analyzed.

FIGS. **3**A through **3**C show that a point mutation in the reactive site loop (RSL) of CrmA inactivates its ability to inhibit ICE.

In FIG. **3**A (top), the reactive site loop sequences of CrmA (SEQ ID NO: 3) and CrmA- (SEQ ID NO: 4) mutant are compared. Amino acid 291 of wild-type CrmA was altered from Thr to Arg by site-directed mutagenesis. The bottom panel shows protein expression in E. Coli as 6xHis fusions and purification as described in the experimental section below. Briefly, 44 ng aliquots of ICE were titrated with the indicated amounts of purified CrmA protein (open squares) or CrmA-mutant (filled circles) protein. Residual ICE activity, expressed as the ratio of the inhibited rate (v_i) to the uninhibited rate (v_o), was determined with a chromogenic ICE substrate and plotted against the quantity of CrmA. ICE activity was abolished by as little as 300 ng of CrmA, whereas no inhibition was detected with CrmA-mutant protein, even using 30 μg, representing a 500-fold molar excess over the enzyme.

FIG. **3**B shows that a CrmA-mutant does not form a complex with ICE. [^{35}S]-Met-labeled CrmA or CrmA-mutant proteins were produced by coupled transcription/translation of their respective genes. The indicated amounts of ICE were added directly to the diluted lysates and incubated as described in the experimental section below, following which samples were resolved by non-denaturing PAGE and the radioactive signals detected using a Phosphorimager. Mutant CrmA failed to form a complex with ICE; indeed, ICE appeared to have no effect on this protein. In comparison, part of the wild-type CrmA formed a complex whereas the rest was cleaved in a manner that typifies the interaction of ICE with CrmA (see Komiyama et al. (1994) *J. Biol. Chem.* 269:19331–19337).

FIG. **3**C shows that the tertiary structures of CrmA and CrmA-mutant proteins are indistinguishable as assessed by transverse urea gradient polyacrylamide gel electrophoresis (TUG-PAGE). [^{35}S]-Met-labeled CrmA or CrmA-mutant proteins were generated by coupled transcription/translation and analyzed by TUG-PAGE as described in the experimental section below. The gels were dried and analyzed using a Phosphorimager to detect each protein's unfolding signature. No difference in the signatures was observed, indicating that the point mutation in CrmA-mutant did not disrupt the protein's tertiary structure.

FIGS. **4**A and **4**B show cleavage of PARP by activated Yama in vitro is inhibitable by CrmA but not by an equivalent amount of CrmA-mutant. Shown in FIG. **4**A are the results of [^{35}S]-Met-labeled Yama that was generated, purified and activated by ICE as described in the experimental section below. Purified, activated Yama was then incubated for 2 hours at 37° C. with 0.586 μg purified PARP in the presence of either buffer (Lane 1), 270 pmol CrmA (Lane 2) or 270 pmol CrmA-mutant (Lane 3) proteins as described below. Following the incubation with PARP, one-fifth of each reaction was analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10. In FIG. **4**B, equivalent amounts of each of the above reactions were subjected to SDS-PAGE and the dried gel analyzed using a Phosphorimager to assess the state of the labeled Yama protein. The open arrows indicated the position of the two major products observed in preparations of activated Yama, and are presumed to correspond to the putative p20 and p11 subunits predicted from the amino acid sequence of Yama.

FIGS. **5**A and **5**B show that CrmA directly interacts with activated Yama but not with Pro-Yama.

FIG. **5**A is a Phosphorimager scan of reaction samples prior to immunoprecipitation analysis. Eighty (80) μl reactions were assembled in which either radiolabeled Pro-Yama (Lanes 1 and 3) or radiolabeled activated Yama (Lanes 2 and 4) were mixed with either 358 pmol CrmA (Lanes 1 and 2) or 358 pmol CrmA-mutant (Lanes 3 and 4) recombinant proteins. Ten (10) microliters of each reaction was resolved by SDS-PAGE and Pro-Yama or activated Yama were detected by phosphorimaging analysis. The filled-in arrow indicates the migration of Pro-Yama (p32), whereas the open arrows indicate the putative p20 and p11 subunits of activated Yama.

FIG. **5**B is an immunoprecipitation of reaction samples with a polyclonal CrmA antiserum. Thirty-five (35) μl of each of the above reactions was subjected to immunoprecipitation using a rabbit polyclonal CrmA antiserum as described below. Precipitates were resolved by SDS-PAGE and radiolabeled proteins detected using a Phosphorimager. The open arrows indicate the putative p20 and p11 subunits of activated Yama.

FIGS. **6**A through **6**C: In FIG. **6**A, CrmA or CrmA-mutant is expressed in stably transfected clones. The left panel shows clonal BJAB cell lines stably transfected with either vector control (BJAB V1, BJAB V4), CrmA (BJAB CrmA2, BJAB CrmA3) or CrmA-mutant (BJAB CrmA-mutant #12, BJAB CrmA-mutant #17) expression constructs that were analyzed by Western blotting using an anti-CrmA antiserum. In the right panel, clonal MCF7 cell lines stably transfected with either vector control (MCF7 V4), CrmA (MCF7 CrmA2, MCF7 CrmA3, MCF7 CrmA4) or CrmA-mutant (CrmA-mutant #1, CrmA-mutant #2) expression constructs were similarly analyzed.

FIG. **6**B shows PARP cleavage to the 85 kDa fragment during Fas-induced apoptosis is inhibited by CrmA but not by CrmA-mutant. Clonal BJAB transfectants not expressing CrmA (BJAB VI, BJAB V4), expressing CrmA (BJAB

CrmA2, BJAB CrmA3) or expressing CrmA-mutant (BJAB CrmA-mutant #12, BJAB CrmA-mutant #17) were treated with agonist anti-Fas (250 ng/ml) antibody for the indicated time periods and lysates were prepared and analyzed by Western blot using the anti-PARP monoclonal antibody C-2-10.

FIG. **6**C shows PARP cleavage to the 85 kDa fragment during TNF-induced apoptosis is inhibited by CrmA but not by CrmA-mutant. Clonal MCF7 transfectants not expressing CrmA (MCF7 V4, MCF7 CrmA2), expressing CrmA (MCF7 CrmA3, MCF7 CrmA4) or expressing CrmA-mutant (MCF7 CrmA-mutant #1, MCF7 CrmA-mutant #2) were treated with TNF (40 ng/ml) for the indicated time periods and lysates prepared and analyzed by Western blot using the anti-PARP monoclonal antibody C-2-10.

FIGS. **7**A–**7**C show that CrmA, but not CrmA-mutant, blocks Fas-and TNF-induced cell death. In FIG. **7**A, MCF7 stably transfected clones were either kept untreated (UnRx) or treated with TNF (40 ng/ml) for 18 hours following which cells were fixed and stained with propidium iodide and nuclear morphology examined by confocal microscopy. CrmA afforded significant protection from TNF-induced apoptosis, whereas both vector-transfected and CrmA-mutant expressing lines were sensitive to TNF-induced apoptosis. In FIG. **7**B, the indicated BJAB stably transfected clones were quantitatively assessed for their sensitivity to Fas-induced PCD using an acridine orange-based apoptosis assay as described below. CrmA-mutant expressing cell lines were uniformly sensitive, whereas CrmA expression afforded significant protection. In FIG. **7**C, the indicated MCF7 stably transfected clones were quantitatively assessed for their susceptibility to TNF-induced cell death as described in the experimental section below.

FIG. **8**A shows TNF and anti-Fas+CHX induce apoptosis in MCF7 cells. MCF7 cells were treated with TNF or anti-Fas+CHX as described in the experimental section below. Upper row: Nuclei of untreated, TNF treated, or anti-Fas+CHX treated MCF7 cells stained with propidium iodide and visualized by laser-scanning confocal microscopy. Arrows indicate examples of apoptotic nuclei. Lower row: Transmission electron microscopy of untreated, TNF treated or anti-Fas+CHX treated MCF7 cells. FIG. **8**B shows that anti-Fas antibody induces apoptosis in BJAB cells. BJAB cells were treated with anti-Fas antibody as described below. The figure shows fluorescence microscopy of untreated or anti-Fas treated BJAB cells stained with acridine orange. Arrows indicate examples of apoptotic nuclei. Inset: Laser scanning confocal microscopy of untreated or anti-Fas treated BJAB cells stained with acridine orange.

FIGS. **9**A–**9**C show inhibition of apoptosis by CrmA in pooled populations of transfectants. Pooled populations of the vector or CrmA transfected cells indicated were analyzed for sensitivity to TNF- or Fas-mediated apoptosis.

FIGS. **10**A–**10**D show inhibition of TNF and anti-Fas+CHX induced apoptosis of MCF7 cells by CrmA. FIG. **10**A: Sensitivities of MCF7 vector transfected clones (V1–V5) or CrmA transfected clones (CrmA1 to CrmA5) to TNF and anti-Fas+CHX induced cell death assessed by propidium iodine apoptosis assay. Insert: Sensitivity of selected clones to TNF and anti-Fas+CHX induced cell death by MTT conversion assay. FIG. **10**B: Northern analysis of corresponding cell lines to detect CrmA transcript. and Northern analysis to detect β-actin transcript loading of RNA.

FIG. **10**C shows CrmA-mediated inhibition of apoptosis induced by increasing doses of anti-Fas antibody.

FIG. **10**D shows CrmA inhibits apoptosis induced by increasing doses of anti-Fas antibody. The acridine orange apoptosis assay was used to analyze the sensitivity of vector transfected (BJAB V1) or CrmA-transfected (BJAB CrmA2, BJAB CrmA3) clones to apoptosis induced by the indicated doses of anti-Fas antibody.

FIGS. **11**A and **11**B show inhibition of anti-Fas induced apoptosis in BJAB cells by CrmA. FIG. **11**A: Analyses of sensitivity of BJAB vector transfected clones (V1–V6) or CrmA transfected clones (CrmA1–CrmA10) to anti-Fas induced apoptosis. Cell death was assessed by acridine orange apoptosis assay. Insert: Sensitivity of selected clones to anti-Fas induced cell death assessed by MTT conversion assay. FIG. **11**B: Northern analysis of corresponding cell lines for detection of expression of CrmA transcript. and: Northern analysis to detect β-actin transcript to assess loading RNA.

FIG. **11**C shows CrmA inhibits apoptosis by increasing doses of anti-Fas antibody. The acridine orange apoptosis assay was used to analyze the sensitivity of vector transfected (BJAB V1) or CrmA-transfected (BJAB CrmA2, BJAB CrmA3) clones to apoptosis induced by the indicated doses of anti-Fas antibody.

FIGS. **12**A and **12**B (SEQ ID NO: 5 and SEQ ID NO: 6) shows the nucleic acid sequence and corresponding amino acid sequence of the cowpox virus CrmA gene and gene product.

FIGS. **13**A and **13**B show that wild type CrmA but not mutant CrmA, inhibits CTL-mediated apoptosis. In FIG. **13**A, clonal lines of BJAB cells stably transfected with either a vector control (clones V1 (■) and V4 (●)) or CrmA expression construct (clones CrmA 2 (▲) and CrmA 3 (○)) were used as target cells in a 24-hour PHA-facilitated allogeneic CTL-mediated cytolysis assay based on ^{51}Cr release as described herein. In FIG. **13**B, clonal lines of BJAB cells stably transfected with either a vector control (clone V1 (■)), CrmA (clone CrmA 2 (●)), or mutant CrmA (clones mutant CrmA 12 (▲) and mutant CrmA 17 (○)) expression constructs were similarly loaded with ^{51}Cr and analyzed in the 24-hour CTL-mediated cytolysis assay. Each of th& data points shown in both A and B represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be directly compared between experiments due to variation arising from differences in blood donors, variation in the degree of protection by CrmA relative to vector or mutant CrmA was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

FIGS. **14**A and **14**B show that CrmA completely blocks the Ca^{2+}-independent component of CTL-mediated killing. In FIG. **14**A, BJAB cells stably transfected with either vector (clones V1 (■) and V4 (●)) or CrmA (clones CrmA 2 (Δ) and CrmA 3 (○)) expression constructs were analyzed in the 24-hour (CTL-mediated cytolysis assays in the absence of EGTA as described under herein. In FIG. **14**B, the same cell lines were analyzed in the 24-hour CTL-mediated cytolysis assay in the presence of 10 mM EGTA and supplemented with 4 mM Mg^{2+}, as described herein. Each of the data points shown in both FIGS. **14**A and B represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be compared between experiments due to variations arising from differences in blood donors,

variation in the degree of protection by CrmA relative to vector was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

FIGS. **15**A and **15**B show that CrmA does not block the Ca^{2+}-dependent component of CTL-mediated cytolysis. FIG. **15**A shows BJAB cells stably transfected with either vector (clones V1 and V4) or CrmA (clones CrmA 2 and CrmA 3) expression constructs that were analyzed in a 4-hour CTL-mediated cytolysis assay in the absence of EGTA as described herein. In FIG. **15**B, the same cell lines were analyzed in a 4-hour CTL-mediated cytolysis assay except in the presence of 10 mM EGTA and supplemented with 4 mM Mg^{2+} as described herein. Each of the data points shown in both Figures A and B represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be compared between experiments due to variation arising from differences in blood donors, variation in the degree of protection by CrmA relative to vector was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

FIG. **16** shows that CrmA blocks CTL-mediated DNA fragmentation. BJAB cells stably transfected with either vector (clones V1 (■) and V4 (●)) or CrmA (clones CrmA 2 (Δ) and CrmA 3 (□) expression constructs were labeled with [methyl-^{3}H]thymidine and DNA fragmentation induced by a 4-hour incubation with CTLs as described under herein. Each data point represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Two independent experiments yielded similar results, and the degree of protection by CrmA relative to vector varied by less than 5% between experiments. 100% DNA fragmentation corresponded to absolute values between 400 and 1000 cpm depending upon the individual experiment.

FIGS. **17**A and **17**B show activation of either Fas or TNF receptor induces U1-70 kDa cleavage. In FIG. **17**A, BJAB cells were either left untreated (UnRx) or treated with agonist anti-Fas monoclonal antibody (250 ng/ml) for the indicated time periods, and cell lysates were prepared and analyzed by Western blotting using a U1-70 kDa reactive antiserum as described herein. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis. In FIG. **17**B, MCF7 cells were either left untreated (UnRx) or treated with TNF (40 ng/ml) for the indicated time periods, and cell lysates were similarly analyzed. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis.

FIGS. **18**A through **18**C show that CrmA, but not mutant CrmA, blocks Fas-induced cleavage of U1-70 kDa. In FIG. **18**A, clonal BJAB cell lines not expressing CrmA (V4, V1, CrmA5) were either left untreated (UnRx) or treated with agonist anti-Fas antibody (250 ng/ml) for the indicated time periods, and lysates were prepared and analyzed by Western blotting using a U1-70 kDa reactive antiserum as described herein. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis. In FIG. **18**B, clonal BJAB transfectants expressing CrmA (CrmA2, CrmA3) were either left untreated (UnRx) or treated with agonist anti-Fas antibody (250 ng/ml) for the indicated time periods and similarly analyzed. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis. In FIG. **18**C, clonal BJAB transfectants expressing mutant CrmA (mutant CrmA/clone #12, mutant CrmA/clone #17) were either left untreated (UnRx) or treated with anti-Fas antibody (250 ng/ml) for the indicated time periods and similarly analyzed. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis.

FIGS. **19**A through **19**C, show that CrmA, but not mutant CrmA, blocks TNF-R-induced cleavage of U1-70 kDa. In **19**A, clonal MCF7 transfectants not expressing CrmA (V4, CrmA2) were either left untreated or treated with TNF (40 ng/ml) for the indicated time periods, and lysates were prepared and analyzed by Western blotting using a U1-70 kDa reactive antiserum as described herein. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis. In **19**B, clonal MCF7 transfectants expressing CrmA (CrmA3, CrmA4) were either left untreated (UnRx) or treated with TNF (40 ng/ml) for the indicated time periods and similarly analyzed. The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis. In **19**C, clonal MCF7 transfectants expressing mutant CrmA (clone #1, clone #2) were either left untreated (UnRx) or treated with TNF (40 ng/ml) for the indicated time periods and similarly analyzed.

The arrow designated "b" indicates a background protein that cross-reacts with the antiserum but is not cleaved during apoptosis.

FIG. **20** shows that mammalian Yama is activated by apoptotic stimuli, e.g., staurosporine. Jurkat cells are either left untreated or treated with 2 μM staurosporine or 200 ng/ml anti-APO-1 antibody for 3 hours. Cytosolic extracts from 10×10^6 Jurkat cells were analyzed by SDS-PAGE and subsequently immunoblotted with antibodies directed against the 17 kDa and 12 kDa subunits of Yama. Similar results were obtained using BJAB or CEM cells. Intermediate forms of the 17 kDa subunit of Yama were also observed (specific only to antibody directed against the 17 kDa subunit) likely containing the pro-domain.

FIGS. **21** A through 21 D show that bcl-2 and bcl-xL function upstream of Yama. FIG. **21**A showns expression of bcl-2 and bcl-xL in Jurkat cell lines. Jurkat cells were transfected with control vector, pEBs-bcl-xL, or pEBs-bcl-2 and selected with hygromycin, SDS-PAGE and immunoblotting were done as described herein. FIG. **21**B shows bcl-2 and bcl-xL prevent staurosporine-induced cell death but not Fas/APO-1-induced cell death. Jurkat cells (2×10^6) were left untreated, treated with staurosporine (2 μM) for 18 hours, or treated with anti-APO-1 antibody (200 ng/ml) for 6 hours. Cells were then analyzed for DNA fragmentation as described herein. Additionally, nuclear morphology assessed by acridine orange staining yielded similar results. FIG. **21** C shows staurosporine-induced induced cleavage of the death substrate, poly(ADP-ribose) polymerase is blocked by bcl-2 and bcl-xL. Jurkat cells were left untreated, treated with 2 μM staurosporine, or treated with 200 ng/ml of anti-APO-1 antibody for 6 hours and analyzed for PARP cleavage as previously described herein. FIG. **21**D shows that bcl-2 and bcl-xL block starurosporine-induced Yama activation. Jurkat cells were left untreated, treated with 2 μM staurosporine for 3 hours, or treated with 200 ng/ml anti-APO-1 antibody for 1.5 hours and analyzed as in FIG. **2**A.

FIGS. **22**A through **22**D show that the CrmA target is upstream of Yama. In **22**A, Jurkat cells were transfected with control vector or pZEM-CrmA and selected with neomycin.

Seven anti-APO-1 resistant clones were identified and pooled. SDS-PAGE and immunoblotting were done as described herein. In **22**B, CrmA prevents Fas/APO-1-induced cell death but not staurosporine-induced cell death. Jurkat cells (2×10^6) were left untreated, treated with staurosporine (2 μM) for 18 hours, or treated with anti-APO-1 antibody (200 ng/ml) for 6 hours. Cells were then analyzed for DNA fragmentation as described herein. Additionally, nuclear morphology assessed by acridine orange staining yielded similar results. In **22**C, Fas/APO-1-, but not staurosporine-induced cleavage of PARP is blocked by CrmA. Jurkat cells were left untreated, treated with 2 μM staurosporine, or treated with 200 ng/ml of anti-APO-1 antibody for 6 hours and analyzed for PARP cleavage as described herein. In **22**D, CrmA blocks Fas/APO-1-induced Yama. Jurkat cells were left untreated, treated with 2 μM staurosporine for 3 hours, or treated with 200 ng/ml anti-APO-1 antibody for 1.5 hours and analyzed.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, various publications, patents and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents and published patent applications are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

As is known to those of skill in the art, apoptosis is an active process of gene-directed cellular self-destruction. This invention provides compositions and methods for preventing or inhibiting apoptosis in a suitable cell or a population of suitable cells. In one embodiment, the method comprises introducing into the cell or cells an effective amount of a nucleic acid molecule coding for a gene product having CrmA biological activity. The method also can be practiced using the gene product itself. It is important to note that the method of this invention inhibits apoptosis even in the presence of apoptotic-inducing agents, such as receptor ligands, e.g., anti-TCR, tumor necrosis factor (TNF), HIV, SIV cytotoxic T-lymphocytes (CTL), or anti-Fas antibody. Accordingly, this method provides an improvement over prior art methods wherein apoptosis can be inhibited by interfering with the induction pathway at the level of ligand induction, such as by providing antibodies or anti-ligand antibodies to interfere with the binding of the ligand to its cell surface receptor. However, this invention can be combined with the use of such prior art methods to inhibit apoptosis. It also can be suitably combined with the administration of bcl-2 type agents, nucleic acids and the like, that also have been shown to inhibit apoptosis. (Lacronique, V. et al. (1996) *Nature Medicine* 2(1):80–86).

This invention also provides a purified or recombinantly produced protease, designated Yama, which is involved in the mammalian cell death pathway as well as a nucleic acid molecule that encodes this protease. It has been demonstrate that purified Yama is a zymogen that, upon activation, assumes a proteolytically competent form that cleaves poly (ADP-ribose) polymerase (PARP) to the signature 85 kDa apoptotic fragment. PARP has been identified as a death substrate that is specifically cleaved during apoptosis. Kaufmann et al. (1989) *Cancer Res.* 49:5870–5878 and (1993) 53:3976–3985 report that the 116 kDa nuclear protein was specifically cleaved to produce an 85 kDa fragment in many forms of PCD, including that induced by chemotherapeutic drugs in cell lines and by dexamethasone in thymocytes. It was later reported by Lazebnik in *Nature* (1994) 371:346–347, in a cell-free system that cleavage occurred C-terminal to Asp, and that the protease responsible resembled ICE in its susceptibility to chemical inhibitors but was distinct from ICE, since purified ICE did not cleave PARP. Yama is further characterized in that its proteolytic activity and apoptotic activity are inhibited by purified CrmA but not by an equivalent quantity of an inactive point mutant of CrmA. As shown in detail in the experimental section below, CrmA blocked the proteolytic cleavage of PARP in cells induced to undergo apoptosis.

Definitions

The terms "proteins", "peptides" and "polypeptides" are used interchangeably and are intended to include purified and recombinantly produced molecules containing amino acids linearly coupled through peptide bonds. The amino acids of this invention can be in the L or D form so long as the biological activity of the polypeptide is maintained. For example, the protein can be altered so as to be secreted from the cell for recombinant production and purification. The proteins of this invention also include proteins that are post-translationally modified by reactions that include glycosylation, acetylation and phosphorylation. Such polypeptides also include analogs, alleles and allelic variants that can contain amino acid derivatives or non-amino acid moieties that do not affect the biological or functional activity of the protein as compared to wild-type or naturally occurring protein. The term amino acid refers both to the naturally occurring amino acids and their derivatives, such as TyrMe and PheCl, as well as other moieties characterized by the presence of both an available carboxyl group and an amine group. Non-amino acid moieties that can be contained in such polypeptides include, for example, amino acid mimicking structures. Mimicking structures are those structures that exhibit substantially the same spatial arrangement of functional groups as amino acids but do not necessarily have both the α-amino and α-carboxyl groups characteristic of amino acids.

"Muteins" are proteins or polypeptides that have minor changes in amino acid sequence caused, for example, site-specific mutagenesis or other manipulations; by errors in transcription or translation; or which are prepared synthetically by rational design. These minor alterations result in amino acid sequences wherein the biological activity of the protein or polypeptide is altered as compared to wild-type or naturally occurring polypeptide or protein. Examples of muteins include the CrmA mutant and the Yama mutant described herein.

As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α-amino group of another amino acid.

As used herein, the term "hydrophobic" is intended to include those amino acids, amino acid derivatives, amino acid mimics and chemical moieties that are non-polar. Hydrophobic amino acids include Phe, Val, Trp, Ile and Leu. As used herein, the term "positively charged amino acid" refers to those amino acids, amino acid derivatives, amino acid mimics and chemical moieties that are positively charged. Positively charged amino acids include, for example, Lys, Arg and His.

"Purified" when referring to a protein or polypeptide, is distinguishable from native or naturally occurring proteins or polypeptides because they exist in a purified state. These "purified" proteins or polypeptides, or any of the intended variations as described herein, shall mean that the compound or molecule is substantially free of contaminants normally

associated with the compound in its native or natural environment. The terms "substantially pure" and "isolated" are not intended to exclude mixtures of polynucleotides or polypeptides with substances that are not associated with the polynucleotides or polypeptides in nature.

"Native" polypeptides, proteins, or nucleic acid molecules refer that those recovered from a source occurring in nature or "wild-type".

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use in vitro, in vivo or ex vivo.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, *Remington's Pharm. Sci.*, 15th Ed. (Mack Publ. Co., Easton (1975)).

The term "nucleic acid" means single and double stranded DNA, cDNA, genome-derived DNA, and RNA, as well as the positive and negative strand of the nucleic acid that are complements of each other, including anti-sense RNA. A "nucleic acid molecule" is a term used interchangeably with "polynucleotide" and each refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. It also includes known types of modifications, for example labels which are known in the art (e.g., Sambrook, et al. (1989) infra.), methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, intemucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl carbamate, etc.), those containing pendant moieties, such as for example, proteins (including, e.g., nuclease, toxins, antibodies, signal peptides, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide. The polynucleotide can be chemically or biochemically modified or contain non-natural or derivatized nucleotide bases. The nucleotides may be complementary to the mRNA encoding the polypeptides. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. Recombinant polynucleotides comprising sequences otherwise not naturally occurring are also provided by this invention, as are alterations of wild type polypeptide sequences, including but not limited to, those due to deletion, insertion, substitution of one or more nucleotides or by fusion to other polynucleotide sequences.

A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well-known to those skilled in the art, it can be transcribed and/or translated to produce a polypeptide or mature protein. Thus, the term polynucleotide shall include, in addition to coding sequences, processing sequences and other sequences that do not code for amino acids of the mature protein. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

The term "recombinant" polynucleotide or DNA refers to a polynucleotide that is made by the combination of two otherwise separated segments of sequence accomplished by the artificial manipulation of isolated segments of DNA by genetic engineering techniques or by chemical synthesis. In so doing one may join together DNA segments of desired functions to generate a desired combination of functions.

An "analog" of DNA, RNA or a polynucleotide, refers to a macromolecule resembling naturally occurring polynucleotides in form and/or function (particularly in the ability to engage in sequence-specific hydrogen bonding to base pairs on a complementary polynucleotide sequence) but which differs from DNA or RNA in, for example, the possession of an unusual or non-natural base or an altered backbone. See for example, Uhlmann et al. (1990) *Chemical Reviews* 90:543–584.

"Isolated" when referring to a nucleic acid molecule, means separated from other cellular components normally associated with native or wild-type DNA or RNA intracellularly.

"Hybridization" refers to hybridization reactions can be performed under conditions of different "stringency". Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, Sambrook, et al., infra. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25° C., 37° C., 50° C., and 68° C.; buffer concentrations of 10×SSC, 6×SSC, 1×SSC, 0.1×SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours and washes of increasing duration, increasing frequency, or decreasing buffer concentrations.

"T_m" is the temperature in degrees Centigrade at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in an antiparallel direction by Watson-Crick base paring dissociates into single strands under the conditions of the experiment. T_m may be predicted according to standard formula; for example:

$$T_m = 81.5 + 16.6 \log [Na+] + 0.41(\%G/C) - 0.61(\%F) - 600/L$$

where Na^+ is the cation concentration (usually sodium ion) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

A "stable duplex" of polynucleotides, or a "stable complex" formed between any two or more components in a biochemical reaction, refers to a duplex or complex that is sufficiently long-lasting to persist between the formation of the duplex or complex, and its subsequent detection. The duplex or complex must be able to withstand whatever conditions exist or are introduced between the moment of formation and the moment of detection, these conditions being a function of the assay or reaction which is being performed. Intervening conditions which may optionally be present and which may dislodge a duplex or complex include washing, heating, adding additional solutes or solvents to the reaction mixture (such as denaturants), and competing with additional reacting species. Stable duplexes or complexes may be irreversible or reversible, but must meet the other requirements of this definition. Thus, a transient complex may form in a reaction mixture, but it does not constitute a stable complex if it dissociates spontaneously or as a result of a newly imposed condition or manipulation introduced before detection.

When stable duplexes form in an antiparallel configuration between two single-stranded polynucleotides, particularly under conditions of high stringency, the strands are essentially "complementary". A double-stranded polynucleotide can be "complementary" to another polynucleotide, if a stable duplex can form between one of the strands of the first polynucleotide and the second. A complementary sequence predicted from the sequence of a single stranded polynucleotide is the optimum sequence of standard nucleotides expected to form hydrogen bonding with the single-stranded polynucleotide according to generally accepted base-pairing rules.

A "sense" strand and an "antisense" strand when used in the same context refer to single-stranded polynucleotides which are complementary to each other. They may be opposing strands of a double-stranded polynucleotide, or one strand may be predicted from the other according to generally accepted base-pairing rules. Unless otherwise specified or implied, the assignment of one or the other strand as "sense" or "antisense" is arbitrary.

A linear sequence of nucleotides is "identical" to another linear sequence, if the order of nucleotides in each sequence is the same, and occurs without substitution, deletion, or material substitution. It is understood that purine and pyrimidine nitrogenous bases with similar structures can be finctionally equivalent in terms of Watson-Crick base-pairing; and the inter-substitution of like nitrogenous bases, particularly uracil and thymine, or the modification of nitrogenous bases, such as by methylation, does not constitute a material substitution. An RNA and a DNA polynucleotide have identical sequences when the sequence for the RNA reflects the order of nitrogenous bases in the polyribonucleotide, the sequence for the DNA reflects the order of nitrogenous bases in the polydeoxyribonucleotide, and the two sequences satisfy the other requirements of this definition. Where at least one of the sequences is a degenerate oligonucleotide comprising an ambiguous residue, the two sequences are identical if at least one of the alternative forms of the degenerate oligonucleotide is identical to the sequence with which it is being compared. For example, AYAAA is identical to ATAAA, if AYAAA is a mixture of ATAAA and ACAAA.

When comparison is made between polynucleotides, it is implicitly understood that complementary strands are easily generated, and the sense or antisense strand is selected or predicted that maximizes the degree of identity between the polynucleotides being compared. For example, where one or both of the polynucleotides being compared is double-stranded, the sequences are identical if one strand of the first polynucleotide is identical with one strand of the second polynucleotide. Similarly, when a polynucleotide probe is described as identical to its target, it is understood that it is the complementary strand of the target that participates in the hybridization reaction between the probe and the target.

A linear sequence of nucleotides is "essentially identical" or the "equivalent" to another linear sequence, if both sequences are capable of hybridizing to form duplexes with the same complementary polynucleotide. It should be understood, although not always explicitly stated that Applicants refer to a specific nulceic acid molecule, its equivalents are also intended. Sequences that hybridize under conditions of greater stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, a polynucleotide region of about 25 residues is essentially identical to another region, if the sequences are at least about 80% identical; more preferably, they are at least about 90% identical; more preferably, they are at least about 95% identical; still more preferably, the sequences are 100% identical. A polynucleotide region of 40 residues or more will be essentially identical to another region, after alignment of homologous portions if the sequences are at least about 75% identical; more preferably, they are at least about 80% identical; more preferably, they are at least about 85% identical; even more preferably, they are at least about 90% identical; still more preferably, the sequences are 100% identical.

In determining whether polynucleotide sequences are essentially identical, a sequence that preserves the functionality of the polynucleotide with which it is being compared is particularly preferred. Functionality can be determined by different parameters. For example, if the polynucleotide is to be used in reactions that involve hybridizing with another polynucleotide, then preferred sequences are those which hybridize to the same target under similar conditions. In general, the T^m of a DNA duplex decreases by about 10° C. for every 1% decrease in sequence identity for duplexes of 200 or more residues; or by about 50° C. for duplexes of less than 40 residues, depending on the position of the mismatched residues (see, e.g., Meinkoth et al.). Essentially identical or equivalent sequences of about 100 residues will generally form a stable duplex with each other's respective complementary sequence at about 20° C. less than T_m; preferably, they will form a stable duplex at about 15° C. less; more preferably, they will form a stable duplex at about 10° C. less; even more preferably, they will form a stable duplex at about 5° C. less; still more preferably, they will form a stable duplex at about T_m. In another example, if the polypeptide encoded by the polynucleotide is an important part of its functionality, then preferred sequences are those which encode identical or essentially identical polypeptides. Thus, nucleotide differences which cause a conservative amino acid substitution are preferred over those which cause a non-conservative substitution, nucleotide differences which do not alter the amino acid sequence are more preferred, while identical nucleotides are even more preferred. Insertions or deletions in the polynucleotide that result in insertions or deletions in the polypeptide are preferred over those that result in the down-stream coding region being rendered out of phase; polynucleotide sequences comprising no insertions or deletions are even more preferred. The relative importance of hybridization properties and the encoded polypeptide sequence of a polynucleotide depends on the application of the invention.

A polynucleotide has the same characteristics or is the equivalent of another polynucleotide if both are capable of forming a stable duplex with a particular third polynucleotide under similar conditions of maximal stringency. Preferably, in addition to similar hybridization properties, the polynucleotides also encode essentially identical polypeptides.

"Conserved" residues of a polynucleotide sequence are those residues which occur unaltered in the same position of two or more related sequences being compared. Residues that are relatively conserved are those that are conserved amongst more related sequences than residues appearing elsewhere in the sequences.

"Related" polynucleotides are polynucleotides that share a significant proportion of identical residues.

As used herein, a "degenerate" oligonucleotide sequence is a designed sequence derived from at least two related

originating polynucleotide sequences as follows: the residues that are conserved in the originating sequences are preserved in the degenerate sequence, while residues that are not conserved in the originating sequences may be provided as several alternatives in the degenerate sequence. For example, the degenerate sequence AYASA may be designed from originating sequences ATACA and ACAGA, where Y is C or T and S is C or G. Y and S are examples of "ambiguous" residues. A degenerate segment is a segment of a polynucleotide containing a degenerate sequence.

It is understood that a synthetic oligonucleotide comprising a degenerate sequence is actually a mixture of closely related oligonucleotides sharing an identical sequence, except at the ambiguous positions. Such an oligonucleotide is usually synthesized as a mixture of all possible combinations of nucleotides at the ambiguous positions. Each of the oligonucleotides in the mixture is referred to as an "alternative form".

A polynucleotide "fragment" or "insert" as used herein generally represents a sub-region of the full-length form, but the entire full-length polynucleotide may also be included.

Different polynucleotides "correspond" to each other if one is ultimately derived from another. For example, messenger RNA corresponds to the gene from which it is transcribed. cDNA corresponds to the RNA from which it has been produced, such as by a reverse transcription reaction, or by chemical synthesis of a DNA based upon knowledge of the RNA sequence. cDNA also corresponds to the gene that encodes the RNA. Polynucleotides also "correspond" to each other if they serve a similar function, such as encoding a related polypeptide, in different species, strains or variants that are being compared.

A "probe" when used in the context of polynucleotide manipulation refers to an oligonucleotide which is provided as a reagent to detect a target potentially present in a sample of interest by hybridizing with the target. Usually, a probe will comprise a label or a means by which a label can be attached, either before or subsequent to the hybridization reaction. Suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes.

A "primer" is an oligonucleotide, generally with a free 3'-OH group, that binds to a target potentially present in a sample of interest by hybridizing with the target, and thereafter promotes polymerization of a polynucleotide complementary to the target.

Processes of producing replicate copies of the same polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "amplification" or "replication". For example, single or double-stranded DNA may be replicated to form another DNA with the same sequence. RNA may be replicated, for example, by an RNA-directed RNA polymerase, or by reverse-transcribing the DNA and then performing a PCR. In the latter case, the amplified copy of the RNA is a DNA with the identical sequence.

A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using one or more primers, and a catalyst of polymerization, such as a reverse transcriptase or a DNA polymerase, and particularly a thermally stable polymerase enzyme. Generally, a PCR involves reiteratively forming three steps: "annealing", in which the temperature is adjusted such that oligonucletide primers are permitted to form a duplex with the polynucleotide to be amplified; "elongating", in which the temperature is adjusted such that oligonucleotides that have formed a duplex are elongated with a DNA polymerase, using the polynucleotide to which they've formed the duplex as a template; and "melting", in which the temperature is adjusted such that the polynucleotide and elongated oligonucleotides dissociate. The cycle is then repeated until the desired amount of amplified polynucleotide is obtained. Methods for PCR are taught in U.S. Pat. Nos. 4,683,195 (Mullis) and 4,683,202 (Mullis et al.).

Elements within a gene include but are not limited to promoter regions, enhancer regions, repressor binding regions, transcription initiation sites, ribosome binding sites, translation initiation sites, protein encoding regions, introns and exons, and termination sites for transcription and translation. An "antisense" copy of a particular polynucleotide refers to a complementary sequence that is capable of hydrogen bonding to the polynucleotide and can therefor, be capable of modulating expression of the polynucleotide. These are DNA, RNA or analogs thereof, including analogs having altered backbones, as described above. The polynucleotide to which the antisense copy binds may be in singe-stranded form or in double-stranded form.

As used herein, the term "operatively linked" means that the DNA molecule is positioned relative to the necessary regulation sequences, e.g., a promoter or enhancer, such that a promoter will direct transcription of RNA off the DNA molecule in a stable or transient manner.

"Vector" means a self-replicating nucleic acid molecule that transfers an inserted nucleic acid molecule into and/or between host cells. The term is intended to include vectors that function primarily for insertion of a nucleic acid molecule into a cell, replication vectors that function primarily for the replication of nucleic acid and expression vectors that function for transcription and/or translation of the DNA or RNA. Also intended are vectors that provide more than one of the above functions.

"Host cell" is intended to include any individual cell or cell culture that can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

A "suitable cell" for the purposes of this invention is one that includes but is not limited to a cell expressing the Fas receptor, e.g., a bone marrow cell, an endothelial cell, a breast carcinoma cell, a fibroblast cell, an epithelial cell, an epithelial tumor cell (see Spriggs, D. R. et al. (1988) *J. Clin. Inves.* 81:455–460), a T cell (TCR^+, $CD8^+$or $CD4^+$ T cells), a peripheral blood lymphocyte, leukocyte, and mixed leukocyte cultures (MLC), a B-lymphoma cell (ATCC, A202J), a colon cell, a small intestine cell, an ovarian cell, a testis cell, a prostate cell, a thymic cell, a spleen cell, a kidney cell, a liver cell, a lung cell, a brain cell and monocytes. Because the Fas (APO-1/CD95) cell surface receptor is a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily, any cell having a receptor of this family is intended to be encompassed by the scope of this invention. Fas and TNF receptor expression also have been identified on numerous tissues, see for example Watanabe-

Fukunaga et al. (1992) *J. Immun.* 148:1274–1279 and Owen-Schaub, L. B. et al. (1994) *Cancer Res.* 54:1580–1586; Dhein et al. (1995) *Nature* 373:438–441; Brunner et al. (1995) *Nature* 373:441–444; and Ju et al. (1995) *Nature* 373:444–448. Assays for identifying additional "suitable" cells sensitive to induction or activation, e.g., TCR-, TNF- or Fas-related apoptosis, are well-known to those of skill in the art. (See for example, Opipari, et al. *J. Biol. Chem.* (1992) 267:12424–12427; Yonehara et al. *J. Exp. Med.* (1989) 169:1747–1756; Dhein et al. (1995) supra; Brunner et al. (1995) supra and Ju et al. (1995) supra).

Suitable cells or "target cells" for the practice of this method also include, but are not limited to, cells that are induced to PCD by an endogenous agent such as HIV, anti-TCR antibody, TNF and anti-Fas antibody. In one embodiment, these cells constitutively and inducibly express receptors for either or both of the cytokine tumor necrosis factor (TNF) or the cell death transducing receptor Fas or TCR and which has been activated by their respective ligand. Recently, three separate groups have reported that Fas-induced apoptosis is involved in T cell death. Specifically, one group has shown that the Fas receptor, which can transduce a potent apoptotic signal when ligated, is rapidly expressed following activation on T cell hybridomas. It was suggested that the Fas receptor-ligand interaction induces cell death in a cell-autonomous manner. See Dhein et al. (1995) *Nature* 373:438–441; Brunner et al. (1995) *Nature* 373:441–444; and Ju et al. (1995) *Nature* 373:444–448.

The cells can be mammalian cells or animal cells, such as guinea pig cells, rabbit cells, simian cells, mouse cells, rat cells, chicken cells or human cells. They can be continuously cultured or isolated from an animal or human. In a separate embodiment of this invention, neurological cells are specifically excluded.

A "biological equivalent" of a nucleic acid molecule is defined herein as one possessing essential identity with the reference nucleic acid molecule. A fragment of the reference nucleic acid molecule is one example of a biological equivalent.

A "biological equivalent" of a polypeptide or protein, is one that contains the active pentapeptide QACRG (SEQ ID NO: 7), in which the cysteine residue is catalytic and which retains the same characteristic as the reference protein or polypeptide. It also includes fragments of the reference protein or polypeptide.

A "dominant inhibitory" protein or polypeptide is one wherein the cysteine residue has been altered such that the catalytic activity is lost but the protein or polypeptide still binds to its intracellular target. Substitution of alanine or methione, for example, for the catalytic cysteine will abolish the catalytic activity while retaining its binding specificity.

"CrmA biological activity" is to mean having the ability to inhibit Fas-induced apoptosis in the manners set forth herein. A dominant inhibitory Yama protein or polypeptide (as well as the nucleic acid coding for this protein or polypeptide) is one example of a agent having CrmA biological activity.

When applied to apoptosis, the terms "preventing" or "inhibiting" are intended to mean a reduction in the number of cells dying or a prolongation in the survival time of the cell or cells. They also are intended to mean a diminution in the appearance or a delay in the appearance of morphological and/or biochemical changes normally associated with apoptosis. Accordingly, "augmentation" of apoptotic cell death means an increase in the total number of cells dying or reduction of the survival time of the cell. "Augmentation" also means a reduction in the time to the appearance of the morphological and/or biochemical changes normally associated with apoptosis after contacting the cells with the apoptotic agent. Apoptosis has been equated with programmed cell death (PCD) and can be detected and monitored by a number of morphological and biochemical changes. The methods that are useful to monitor and detect these changes include light microscopy, a measurement between potential and actual tumor doubling times, loss of radiolabeled DNA precursors, measurement of DNA fragmentation, or measurement by FCM. These methods are reviewed Vermes and Haanen, "Apoptosis and Programmed Cell Death in Health and Disease" *Adv. in Clin. Chem.* (1994) 31:177–246, and the references cited therein. Light microscopy and the measurement of the potential tumor doubling time versus the actual tumor volume doubling time are most applicable in mammalian pathology. "Inhibition" when used in this context, means a reduction in the number of cells undergoing apoptosis or PCD or an increase in survival time or growth rate of a cell or population of treated cells as compared to a control population. "Augmentation" means an increase in the number of cells undergoing apoptosis or PCD or a decrease in survival time or growth rate of a cell or population of treated cells as compared to a control population. A "treated cell" is a cell or a population of cells that have been exposed to the protein or antibody or have inserted therein by any number of methods a nucleic acid molecule of this invention.

As used herein, a Pro-Yama protein, activated Yama, p20 Yama and p11 Yama are intended to include wild-type or naturally occurring protein, as well as muteins (e.g., dominant inhibitory Yama), analogs, biological equivalents and fragments thereof. In some embodiments, the term also includes anti-Yama antibodies and anti-idiotypic antibodies as well as chimeras including the antibody or a fragment of the antibody.

Proteins and Polypeptides

This invention provides proteins or polypeptides, purified from a native environment or recombinantly obtained, designated Pro-Yama, p20 Yama, p11 Yama, activated Yama, and mutant Yama as well as their biological equivalents. A biological equivalent is a protein or polypeptide that has the same or essentially similar biological activity as determined by the assays described herein. Unless specifically identified, the term Yama protein or polypeptide is to include all forms and biological equivalents described herein. The proteins and polypeptides can be purified from an animal source such as rat, chicken, human or mouse. The recombinant forms are obtainable from a number of procaryotic and eucaryotic recombinant systems or can be chemically synthesized. This invention further provides a mutant CrmA protein that does not inhibit apoptosis.

Pro-Yama is a zymogen which upon "activation" cleaves PARP to an 85 kDa form. In one embodiment, Pro-Yama has an apparent molecular weight of about 32 kDa as determined on PAGE. In a separate embodiment, it has the 277 amino acid sequence shown in FIG. **1**. Activated Yama consists of two subunits designated herein as consisting of p20 Yama and p11 Yama. The actual molecular weight of the subunits is 17 and 12 kDa, respectively as shown in FIGS. **20** and **21**. Pro-Yama is cleavable by ICE following an aspartic acid to form activated Yama consisting of the p20 and p11 subunits. Accordingly, this invention also provides p20 Yama and p11Yama purified from a native environment or obtained recombinantly. Activated Yama (the p20 Yama and p11 Yama) are heterodimeric polypeptides that, in combination,

are characterized in having the biological or finctional ability to modulate cellular finction associated with Fas receptor pathway such as Fas-associated apoptosis. In particular, p20 Yama and p11 Yama heterodimer promotes apoptosis in a suitable cell, the activity of which is inhibitable by CrmA but not mutant CrmA. They also form an inhibitory complex with CrmA but not mutant CrmA.

In one embodiment of this invention, overexpression of the DNA encoding an activated Yama protein promotes apoptosis. Examples of such proteins include, but are not limited to p20 Yama and p11 Yama. In a separate embodiment, the biological activity of the p20 or p11Yama proteins or their equivalents are inhibitable by CrmA but not the mutant CrmA described herein. The CrmA gene or nucleic acid can be isolated from natural or native sources as described in Pickup et al. (1986) *PNAS* 83:7698–7702. One of skill in the art can determine when and if the biological activity of a protein is inhibitable by CrmA using the method disclosed in Tewari et al. (1995) *J. Biol. Chem.* 270:3255–3260 or the methods disclosed below.

Yama and Yama subunits can be purified from a suitable cell lysate by using epitope tagged versions highly expressed in 293T (ATCC) cells using the method disclosed in Chiang and Roeder (1993) *Peptide Research* 6(2):62–64.

Also provided by this invention are polypeptide fragments of purified or recombinantly produced Pro-Yama, activated Yama, p20 Yama, or p11 Yama or the protein having the amino acid sequence shown in FIG. **1**. These peptides are characterized by either being activated to an apoptotic promoting form (activated Yama) or being able to promote apoptosis in an activated cell. One such fragment is the pentapeptide QACRG (SEQ ID NO: 7), wherein the cysteine is catalytic.

It is understood that functional equivalents of the Yama proteins identified above also are within the scope of this invention. For illustration purposes only, a functional equivalent includes the Yama fusion protein 6xHis-Yama or those containing chemical structures other than amino acids, which functionally mimic the biological activity of any purified Pro-Yama, its allelic variant, activated Yama, purified p20 Yama, purified p11 Yama, the recombinant homologs thereof or the protein having the amino acid sequence shown in FIG. **1** ("analogs") which retain the biological activity of the corresponding purified or recombinant protein or polypeptide. An additional example of an analog is a protein or polypeptide containing a distinct protein or polypeptide joined to Yama or fragments thereof, e.g., a GST fusion protein, the equivalents that vary the primary sequence of protein of this invention from the amino acid sequence provided in FIG. **1**. However, in one embodiment of this invention, the proteins designated CPP32β, Mch2, Ced-3, Nedd-2, TX/ICH2, ICE rel-III and ICE-rel II are specifically excluded. (See Fernandes-Alnemri, et al. (1994) *J. Biol. Chem.* 269:30761–30764).

An agent characterized by having the ability to inhibit the binding of activated Yama or p20 and/or p11 Yama to CrmA is further provided by this invention. Such agents include, but are not limited to, an anti-CrmA antibody or a dominant inhibitory fragment of any of CrmA, or the p20, p11 or Pro-Yama. A "dominant inhibitory fragment" of this invention is intended to include but is not limited to, a mutein that irreversibly binds intracellular CrmA or the p20 and p11 Yama heterodimeric complex, respectively. Examples of dominant inhibitory muteins of Yama are those in which the functionally important cysteine sequence QACRG in p20 and Pro-Yama is mutated to alanine or methionine. These mutants have lost their apoptotic capacity but still bind their substrates thereby blocking endogenous native Yama and its biological activity or function. These Yama muteins can be made by using the amino acid sequence provided in FIG. **1** and a modification of the methods provided herein and Higuchi et al. (1988) infra.

The proteins and polypeptides of this invention are obtainable by a number of processes well-known to those of skill in the art, which include purification, chemical synthesis and recombinant methods. Full length Pro-Yama, activated Yama, p20 Yama and pl Yama proteins can be purified from a Fas^+ cell or tissue lysate using methods such as immunoprecipitation with an appropriate antibody, and standard techniques such as gel filtration, ion-exchange, reversed-phase, and affinity chromatography. For such methodology, see for example Deutscher et al., *Guide to Protein Purification: Methods in Enzymology* (1990) Vol. 182, Academic Press. Accordingly, this invention also provides the processes for obtaining the proteins and polypeptides of this invention as well as the products obtainable and obtained by these processes.

The proteins and polypeptides also can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Applied Biosystems, Inc., Model 430A or 431A, Foster City, Calif. and the amino acid sequence provided in FIG. **1** and FIG. **12**. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this invention also provides a process for chemically synthesizing the proteins of this invention by providing the sequence of the protein (e.g., FIG. **1** for Yama and FIGS. **3**A and **12** for mutant CrmA) and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2d ed. (Cold Spring Harbor Laboratory (1989)) using, for example, the host cell and vector systems described and exemplified below. This invention further provides a process for producing a Pro-Yama, activated Yama, p20 or p11Yama protein, analog, mutein or fragment thereof, by growing a host cell containing a nucleic acid molecule encoding the desired protein, the nucleic acid being operatively linked to a promoter of RNA transcription. The host cell is grown under suitable conditions such that the nucleic acid is transcribed and translated into protein. In a separate embodiment, the protein is further purified.

Also provided by this invention are the proteins described herein conjugated to a detectable agent for use in diagnostic methods. For example, detectably labeled proteins and polypeptides containing the p20 and p11 heterodimeric Yama can be bound to a column and used for the detection and purification of CrmA. They also are useful as immunogens for the production of antibodies as described below. The proteins and fragments of this invention are useful in an in vitro assay system to screen for agents or drugs which either inhibit or augment a Fas-related function such as apoptosis and to test possible therapies for disorders associated with this pathway, e.g., lps, immunosuppression, depletion of $CD4^+$ T cells, and carcinogenesis.

More specifically, the in vitro cellular method comprises providing cell cultures or tissue cultures having either a cell surface receptor that mediates apoptosis such as a TCR, the TNF receptor or the Fas receptor. The cells are cultured

under conditions (temperature, growth or culture medium and gas (CO_2)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. The cells are then exposed to preliminary conditions necessary for apoptosis, for example an effective amount of an inducing agent, e.g., a TCR ligand, HIV, CTL, SIV, TNF, or a Fas ligand such as an anti-Fas antibody is added to the culture. Anti-Fas antibodies and mitogens (ConA) are well-known to those of skill in the art. (Itoh, N. et al. (1991) *Cell* 66:233–243 and Yonehara et al. (1989) *J. Exp. Med.* (1989) 169:1747–1756). These cells are now "induced" to apoptosis. The cells are again cultured under suitable temperature and time conditions. In one embodiment, HIV or SIV is added to the culture. In other embodiments, a drug or agent to be tested is added in varying concentrations at a time that is simultaneous with, prior to, or after the inducing agent.

If the cell constutively express Yama, the addition of Pro-Yama is not required. In other embodiments, Pro-Yama nucleic acid molecule or protein is then added to the culture in an effective amount and under conditions that the cells internalize the nucleic acid or protein. In some embodiments, an effective amount of ICE or ICE nucleic acid is added to activate Pro-Yama. The cells are cultured under suitable temperature and time conditions to induce apoptosis. The cells are separated into two samples. In the first set, the CrmA nucleic acid or protein can be added prior to, simultaneously with, or after, the agent to be tested. The cells are assayed for apoptotic activity using methods well-known to those of skill in the art and described herein. It is apparent to those of skill in the art that at least two separate culture of cells must be treated and maintained as the test population. One is maintained without receiving an inducing agent to determine background release and the second without the receiving the agent to be tested. The second population of cells acts as a control.

The use of the compositions and methods in vitro provides a powerful bioassay for screening for drugs that are agonists or antagonists of CrmA and Yama function in these cells. Thus, one can screen for drugs having similar or enhanced ability to prevent or inhibit apoptosis as CrmA or the ability to induce apoptosis as Yama. One of skill in the art can determine when the method has been successfully performed by noting the absence of apoptotic morphological changes or more simply, by the absence of cell death. The in vitro method further provides an assay to determine if the method of this invention is useful to treat a subject's pathological condition or disease that has been linked to apoptotic cell death in the individual.

For example, a T cell hybridoma cell line such as Jurkat can be stably transfected with the CrmA expression construct, expression vector containing Pro-Yama, activated Yama, CrmA or mutant Yama, or vector alone and clonal cell lines derived. Transfection of Jurkat cell by electroporation can be performed as described in Laherty et al. *J. Biol. Chem.* (1993) 263:5032–5039. The cells are ^{51}Cr-labeled and plated (5×10^5/ml) on untreated or anti-CD3 (available from the cell line 145-2C 11 (ATCC)) treated tissue culture plastic plates. Cells cultured on uncoated cells are used to determine background release. The percentage cell death will be determined at various times after culture by the formula: c.p.m. released from the experimental group minus c.p.m. of background release divided by c.p.m. released by 0.5% Triton X-100 (complete lysis)—c.p.m. of background release. Agents are then added to the culture to determine their effect on apoptosis, with and without exogenously added CrmA, Yama and mutant Yama nucleic acids or proteins. Using the method described above, various agents can be tested for their ability to inhibit, prevent or augment apoptosis.

In a separate embodiment, the T cell line designated CEM (ATCC) is obtained and used because it has been shown to undergo PCD upon infection with HIV. CEM cells are transfected by electroporation with the CrmA expression construct and vector alone as control. Clonal lines are derived and infected at various multiplicity of infection ratios with HIV. Cytopathic effect is assayed by microscopic observation and apoptosis quantitated following propidium iodine staining. Using the method described above, various agents can be tested for their ability to inhibit or prevent apoptosis.

The proteins of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and selects one. However, for the purpose of illustration only, suitable adjuvants include, but are not limited to Freund's Complete and Incomplete, mineral salts and polynucleotides.

This invention also provides a pharmaceutical composition comprising any of a protein, analog, mutein, polypeptide fragment, antibody, antibody fragment or anti-idiotipic antibody of this invention, alone or in combination with each other or other agents, and an acceptable carrier. These compositions are useful in various diagnostic and therapeutic methods.

Nucleic Acids

Nucleic acid molecules and isolated nucleic acid molecules that encode amino acid sequences corresponding to Pro-Yama, activated Yama, Yama mutein, CrmA mutein analog, p20 or p11 Yama polypeptide, antibodies, anti-idiotypic antibodies and antibody fragments, as well as complements of these sequences, are further provided by this invention. In addition to the sequences shown in FIGS. **1**, **3**A and **12**, this invention also provides the anti-sense polynucleotide stand, e.g., antisense RNA. One can obtain an antisense RNA using the sequence provided in FIG. **1**, for example, and the methodology described in Vander Krol et al. (1988) *BioTechniques* 6:958. Unless specifically identified the term "Yama" nucleic acid is to encompass all the Yama nucleic acid molecules described herein.

In one aspect of this invention, the nucleic acid molecule encoding Yama protein or polypeptide is defined to be the entire sequence or parts of the sequence shown in FIG. **1**. Further provided are nucleic acid molecules comprising the oligonucleotide coding for the pentapeptide QACRG (SEQ ID NO: 7), and the dominant inhibitory polypeptides and proteins described herein. Also included within the scope of this invention is the DNA and RNA complements of these nucleic acid molecules.

The invention also encompasses nucleic acid molecules that differ from that of the nucleic acid molecules described above, but which produce the same phenotypic effect, such as an allele. These altered, but phenotypically equivalent nucleic acid molecules are referred to "equivalent nucleic acids." This invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced

therefrom when compared to the nucleic acid molecule herein. This invention further encompasses nucleic acid molecules that hybridize to the nucleic acid molecules of the subject invention under stringent conditions. Also within the scope of this invention are nucleic acids having a sequence altered from that shown in FIG. **1** but produce a protein having enhanced or diminished biological activity.

In one embodiment, specifically excluded are the nucleic acid molecules encoding the proteins designated CPP32B, Mch2, Ced-3, Nedd-2, TX/ICH2, ICE rel-III and ICE-rel II are specifically excluded.

The nucleic acid molecules can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene encoding Yama in a cell. Briefly, this invention further provides a method for detecting a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of Yama by contacting single-stranded nucleic acid molecules with a labeled, single-stranded nucleic acid molecule (a probe or primer) which is complementary to a single-stranded nucleic acid molecule encoding an amino acid sequence that is at least a portion of the Yama protein under conditions permitting hybridization (preferably stringent hybridization conditions) of complementary single-stranded nucleic acid molecules. Hybridized nucleic acid molecules are separated from single-stranded nucleic acid molecules. The hybridized molecules are detected using methods well-known to those of skill in the art and set forth, for example, in Sambrook (1989) supra.

The nucleic acid molecules of this invention can be isolated using the technique described in the experimental section or replicated using PCR (Perkin-Elmer). For example, the sequence can be chemically replicated using PCR (Perkin-Elmer) which in combination with the synthesis of oligonucleotides, allows easy reproduction of DNA sequences. The PCR technology is the subject matter of U.S. Pat. Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and described in *PCR: The Polymerase Chain Reaction* Mullis et al. eds, Birkhauser Press, Boston (1994) and references cited therein. Alternatively, one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, nucleotides, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the nucleic acid into a suitable replication vector and insert the vector into a suitable host cell (a human B cell or BJAB or 293 T cell) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well-known to those of skill in the art. A process for obtaining nucleic acid molecules by this method is further provided herein as well as the nucleic acid molecules so obtained.

RNA can be obtained by using the isolated DNA and operatively linking it to a control region appropriate for the host cell and inserting it into a host cell. A suitable cell for this purpose includes but is not limited to a human B cell, BJAB or 293T cell. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate insertion vector or by electroporation. When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well-known to those of skill in the art, for example, as set forth in Sambrook et al. (1989) supra.

The invention further provides the nucleic acid molecule operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or transient or stable expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct transcription of RNA off the DNA molecule. Examples of such promoters are SP6, T4 and T7. In certain embodiments, cell-specific promoters are used for cell-specific expression of the inserted nucleic acid molecule. Vectors that contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well-known in the art and commercially available. For general methodology and cloning strategies, see *Gene Expression Technology*, Goeddel ed., Academic Press, Inc. (1991) and references cited therein and *Vectors: Essential Data Series* Gacesa and Ramji, eds., John Wiley & Sons, N.Y. (1994), which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession number for various suitable vectors. Preferable, these vectors are capable of transcribing RNA in vitro or in vivo.

Fragments of the polynucleotide shown in FIG. **1** also are encompassed by this invention, preferably at least 8 nucleotides and more preferably having at least 10 or 18 nucleotides. These are useful as hybridization probes and PCR primers.

In one embodiment, these fragments are nucleic acid molecules that encode proteins designated activated Yama or p20 Yama and p11Yama and the pentapeptide QACRG (SEQ ID NO: 7). The nucleic acid molecules encode polypeptides which heterodimerize and bind CrmA and induce apoptosis in an activated cell. This and additional fragments of this invention are useful to code for proteins having diagnostic and therapeutic utilities as described herein as well as probes to identify transcripts of the protein that may or may not be present. These polynucleotide fragments can be prepared, for example, by restriction enzyme digestion of the nucleic acid molecule of FIG. **1** and then labeled with a detectable marker such as a radioisotope using well-known methods. Alternatively, random fragments can be generated using nick translation of the molecule. For methodology for the preparation and labeling of such fragments, see Sambrook et al., *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989). Polynucleotide fragments also are useful to generate novel peptides. These peptides, in turn, are useful as immunogens for the generation of polyclonal and monoclonal antibodies.

As noted above, a nucleic acid molecule of this invention can be operatively linked to a promoter, either an inducible or non-inducible promoter, of RNA transcription. These nucleic acid molecules are useful for the recombinant production of Yama proteins and polypeptides or as vectors for use in gene therapy. Accordingly, this invention also provides a vector (insertion, replication or expression vector) having inserted therein a nucleic acid molecule described above, for example, a viral vector, such as bacteriophage, baculovirus and retrovirus, or cosmids, plasmids, YACS, yeast and other recombinant vectors. Nucleic acid molecules are inserted into vector genomes by methods well-known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the insert

DNA that corresponds to a restriction site in the vector DNA, which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV) for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for in vitro transcription of sense and anti-sense RNA.

An additional example of a vector construct of this invention is a bacterial expression vector including a promoter such as the lac promoter and for transcription initiation, the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) supra). Similarly, a eucaryotic expression vector is a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled using the sequences described herein. In one embodiment of this invention, the expression vector is to be specifically targeted to T cells. For these methods, it intended that the DNA be operatively linked to a promoter that is highly active in T cells. Such promoters include, but are not limited to: IFN-α; IL-2; IL-3; IL-4; IL-5; IL-9; IL-10; TFN-α; GM-CSF; CD4, CD8 and the IL-2 promoter.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce proteins, e.g., Yama proteins and polypeptides and the mutant CrmA described herein. It is implied that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues in vivo because of their high levels of expression and efficient transformation of cells both in vitro and in vivo. When a nucleic acid is inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic cell and the host cell replicates, the protein can be recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well-known methods. See Sambrook et al. (1989) supra. In addition to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic acid can be inserted into the host cell by methods well-known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See Sambrook et al. (1989) supra for this methodology. Thus, this invention also provides a host cell, e.g., a mammalian cell, an animal cell (rat or mouse), a human cell, or a bacterial cell, containing a nucleic acid molecule encoding a Yama protein or polypeptide or an Yama protein or polypeptide or antibody.

Using the host vector system described above, a process of producing and/or obtaining recombinant Yama, analog, mutein, or anti-Yama antibody or active fragments thereof or mutant CrmA is provided by growing the host cells described herein under suitable conditions such that the nucleic acid encoding Yama or anti-Yama protein, polypeptide or antibody is expressed. Suitable conditions can be determined using methods well-known to those of skill in the art, see for example, Sambrook et al., (1989) supra. The recombination products are then purified from the cellular extract. Accordingly, this invention further provides host cells containing exogenously added nucleic acid molecules of this invention as well as processes for recombinantly producing the proteins, polypeptides and antibodies of this invention by performing the above mentioned steps as well as the products so produced.

A vector containing the nucleic acid encoding Yama, activated Yama, anti-Yama protein, Yama antisense RNA, nucleic acid molecule encoding Yama antisense RNA or antibody also is useful for gene therapy to modulate or regulate cellular functions such as apoptosis and immune disorders mediated by the Fas pathway. The term "Fas cellular function" is intended to mean cellular functions that are affected by the binding of the receptor to its extracellular ligands, i.e., alone or in combination with each other. In some instances, for example in a neoplastic or carcinoma cell, it is desirable to augment Fas apoptotic function to induce apoptosis. This can be achieved by introducing into the cell Pro-Yama and an activating agent such as ICE or p20 and p11 heterodimeric Yama protein or nucleic acid molecules encoding polypeptides and proteins having this biological activity. In other instances, it is desirable to down-regulate Fas cellular function. This can be accomplished by introducing into the cell an antibody fragment that is a dominant inhibitor of activated Yama or p20 or p11 Yama, Yama antisense RNA (or the DNA which codes for it) or CrmA protein or the nucleic acid molecules coding for these agents. In addition, anti-sense Yama RNA can be used to inhibit production of the Pro-Yama protein. This therapy will inhibit or disable intracellular Fas signaling and therefore is a useful therapy where apoptotic cell death is to be avoided, such as in an HIV-infected T cell.

When used for gene therapy in vivo or ex vivo, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted nucleic acid molecule. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-incompetent retroviral vector is LNL6 (Miller, A. D. et al. (1989) *BioTechniques* 7:980–990). The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, et al. (1989) *PNAS USA* 86:8912; Bordignon (1989), *PNAS USA* 86:6748–52; Culver, K. (1990), *PNAS USA* 88:3155; and Rill, D. R. (1991) *Blood* 79(10):2694–700. Clinical investigations have shown that there are few or no adverse effects associated with the viral vectors, see Anderson, (1992) *Science* 256:808–13.

Compositions containing the nucleic acid molecules of this invention, in isolated form or contained within a vector or host cell are further provided herein. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

Antibodies

Also provided by this invention is an antibody capable of specifically forming a complex with the proteins, polypep-

tides and nucleic acid molecules of this invention. These include, but are not limited to Pro-Yama, activated Yama, QACRG (SEQ ID NO: 7), dominant inhibitory polypeptides and protein, fragments of Yama such as p20 Yama and p11 Yama, as well as nucleic acid molecules encoding the antibodies. Vectors and host cells containing these nucleic acid molecules also are encompassed by this invention. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, rabbit or human antibodies.

As used herein, an "antibody" or "polyclonal antibody" means a protein that is produced in response to immunization with an antigen or receptor and that reacts with the antigen with an effective specificity and affinity for its intended purpose. The term "monoclonal antibody" means an immunoglobulin derived from a single clone of cells. All monoclonal antibodies derived from the clone are chemically and structurally identical, and specific for a single antigenic determinant. The hybridoma cell lines producing the monoclonal antibodies also are within the scope of this invention.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies, as well as deducing their corresponding nucleic acid sequences, are known in the art, see Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988) U.S. Pat. No. 5,411,749 and Sambrook et al. (1989) supra. The monoclonal antibodies of this invention can be biologically produced by introducing Yarna protein or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas. Accordingly, the hybridoma cells producing the monoclonal antibodies of this invention also are provided.

Thus, using the Yama protein or fragment thereof, and well-known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind Yama.

If a monoclonal antibody being tested binds with a Yama protein or polypeptide, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this invention by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding Yama with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the invention as shown by a decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with Yama protein with which it is normally reactive, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this invention.

The term "antibody" also is intended to include antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described in Steplewski et al. (1985) *Proc. Natl. Acad. Sci.* 82:8653 or Spira et al. (1984) *J. Immunol. Methods* 74:307.

This invention also provides biological active fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody fragments can include, but are not limited to:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule obtained by treating with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $F(ab')_2$, the fragment of the antibody that is obtained by treating with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by at least one disulfide bond;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) SCA, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

A specific example of "biologically active antibody fragment" include the CDR and VH regions of the antibodies. Methods of making these fragments are known in the art, see for example, Harlow and Lane, (1988) supra and Davies et al. (1995) *Bio/Technology* 13(5):475–479.

The antibodies of this invention also can be modified to create chimeric antibodies and humanized antibodies (Oi, et al. (1986) *Bio Techniques* 4(3):214). Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, et al. (1986) *Science* 232:100). An anti-idiotypic antibody is an antibody that recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest. These determinants are located in the hypervariable region of the antibody. It is this region that binds to a given epitope and, thus, it is responsible for the specificity of the antibody. The anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The animal immunized will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the second animal, which are specific for the monoclonal antibodies produced by a single hybridoma that was used to immunize the second animal, it is now possible to identify other clones with similar idiotypes as the antibody of the hybridoma used for immunization.

Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is pos-

sible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies that mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region that is the mirror image of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

As used in this invention, the term "epitope" is meant to include any determinant having specific affinity for the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Also encompassed by this invention are proteins or polypeptides that have been recombinantly produced, biochemically synthesized, chemically synthesized or chemically modified, that retain the ability to bind Pro-Yama, p20 Yama or p11 Yama, or fragments thereof, corresponding native polyclonal or monoclonal antibody.

The antibodies of this invention can be linked to a detectable agent or a hapten. The complex is useful to detect the Fas receptor or Yama protein or fragments in a sample or detect agents that interfere with Yama-Fas receptor binding, using standard immunochemical techniques such as immunohistochemistry as described by Harlow and Lane (1988) supra. Examples of types of immunoassays that can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the enzyme linked immunoassay (ELISA) radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the monoclonal antibodies of the invention can be done utilizing immunoassays that are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

Another technique that may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts avidin, or dinitropherryl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies. See Harlow and Lane (1988) supra.

The monoclonal antibodies of the invention can be bound to many different carriers. Thus, this invention also provides compositions containing the antibodies and another substance, active or inert. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibody, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody of the invention can be done using standard techniques common to those of ordinary skill in the art.

For purposes of the invention, Yama may be detected by the monoclonal antibodies of the invention when present in biological fluids and tissues. Any sample of cells or tissue lysate containing a detectable amount of Yama can be used.

Compositions containing the antibodies, fragments thereof or cell lines which produce the antibodies, are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

Compositions

This invention also provides compositions containing any of the above-metioned proteins, muteins, polypeptides, nucleic acid molecules, vectors, host cells antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnosis and treatment of pathologies associated with the Fas receptor and apoptotic pathway.

Industrial Applicability

The compositions described above provide the components for an assay to screen for agents and pharmaceutical compounds that are agonists or antagonists of Fas-associated apoptosis in a suitable cell. A suitable cell is one that contains the Fas/CD95 or TNF receptor or which is induced to apoptosis or PCD by an endogenous agent such as HIV, CTL, anti-TCR antibody, a Fas agonist, TNF or an anti-Fas antibody. In one embodiment, these cells constitutively and inducibly express receptors for either or both of the cytokine tumor necrosis factor (TNF) or the cell death transducing receptor Fas or TCR and which has been activated by their respective ligand. Recently, three separate groups of investigators have reported that Fas-induced apoptosis is involved in T cell death. Specifically, one group has shown that the Fas receptor, which can transduce a potent apoptotic signal when ligated, is rapidly expressed following activation on T cell hybridomas. It was suggested that the Fas receptor-ligand interaction induces cell death in a cell-autonomous manner. See Dhein et al. (1995) *Nature* 373:438–441; Brunner et al. (1995) *Nature* 373:441–444; and Ju et al. (1995) *Nature* 373:444–448.

For the purpose of illustration only, examples of suitable cells are T lymphocytes (T cells, e.g., TCR^+, $CD4^+$ and $CD8^+$T cells) leukocytes and mixed leukocyte cultures (MLC), B lymphoma cells (e.g., A202J (ATCC)), peripheral blood lymphocytes, colon cells, small intestine cells, an ovarian cells, testis cells, prostate cells, thymic cells, spleen cells, kidney cells, liver cells, neoplastic cells, carcinoma cells, lung cells or brain cells, each from a mammalian species, e.g., mouse, rat, simian or human.

As provided in more detail below, the proteins and fragments thereof are useful in a cell-free and cellular in vitro assay system to screen for agents and pharmaceutical compounds which either inhibit or augment the Fas-receptor pathway and apoptosis and to test possible therapies for disorders associated with this pathway, e.g., lps, immunosuppression, depletion of $CD4^+$T cells, and carcinogenesis. Embryogenesis also can be modulated.

The cell free screen is performed essentially as set forth below. For example, an effective amount of Pro-Yama is activated by incubating with ICE in Reaction Buffer at 37° C. Following activation, the reaction is divided into two parts. Into one part an effective amount of the agent to be tested is added. Into the second part, an effective amount of CrmA is added. Each reaction mix is incubated for approximately 30 minutes. An effective amount of PARP is added to each mix and the solutions are further incubated at 37° C. for an effective amount of time or about 2 hours. Following this incubation, a sample from each reaction mixture is analyzed by immunoblotting with anti-PARP monoclonal antibody such as C-2-10 or by gel electrophoresis to determine if the agent inhibited cleavage of PARP to its characteristic 85 kDa form. The presence of the 85 kDa form is an indication that the agent is not an inhibitory agent and the absence of the 85 kDa form is an indication that the agent is candidate for inhibiting a Fas-associated function such as apoptosis. This method also can be practiced by substitution of an effective amount of U1-70 kDa protein for PARP. Cleavage to the 40 kDa form, which can be detected by the use of an antibody, indicates that the agent is not a candidate for inhibiting apoptosis.

Also encompassed by this invention are the agents detected by these methods, the nucleic acid molecules encoding them and the use of these agents and nucleic acid molecules in the therapeutic methods described herein. As is apparent to those of skill in the art, the above compositions can be combined with instructions for use to provide a kit for a commercially available screen.

The above methods allow one also to screen for drugs having similar or enhanced ability to prevent or inhibit apoptosis as compared to CrmA, for example.

In the cellular in vitro method, suitable cell cultures or tissue cultures are provided. The cells are cultured under conditions (temperature, growth or culture medium and gas (CO_2)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. The cells are then exposed to preliminary conditions necessary for apoptosis, for example an effective amount of an inducing agent, e.g., a TCR ligand, HIV, SIV, TNF, CTL, or a Fas ligand such as an anti-Fas antibody is added to the culture. Anti-Fas antibodies and mitogens (ConA) are well-known to those of skill in the art. (Itoh, N. et al. (1991) *Cell* 66:233–243 and Yonehara et al. (1989) *J. Exp. Med.* 169:1747–1756). These cells are now "induced" to apoptosis. Alternatively, the cells can be contacted with the inducing agent after transfection with the Yama nucleic acid and agent. The cells are again cultured under suitable temperature and time conditions. An effective amount of an agent that is believed to inhibit apoptosis in this system is added to the cell culture. For example, an effective amount of a nucleic acid molecule encoding Pro-Yama, p20 Yama or p11 Yama is contacted with the cell or tissue culture so as to insert the nucleic acid. Alternatively, an effective amount of the polypeptide or protein products is added to the cell culture. The cells are again cultured for expression of the inserted nucleic acid molecule. An effective amount of the agent to be tested is then added to the cell or tissue culture in varying concentrations. In a separate embodiment wherein the cells constitutively express Yama, the method can be practiced without addition to the cells.

Because the activity of activated Yama or p20 and p11 Yama is inhibitable by CrmA, a separate culture of cells that can act as a comparison is cultured under identical conditions as described above, except that CrmA nucleic acid is added to the culture rather than the agent. The CrmA nucleic acid or protein is added the culture in an effective amount and the cells are cultured under suitable temperature and time conditions to inhibit apoptosis. The CrmA nucleic acid or protein can be added prior to, simultaneously with, or after, the inducing agent.

It also is desirable to maintain additional separate cell cultures; one that does not receive an inducing agent to determine background release and another that does not receive the agent to be tested.

Each of the samples of cells is then assayed for apoptotic activity using methods well-known to those of skill in the art and described herein. An example of this screen is provided below.

The compositions provided herein are useful to modulate the Fas receptor pathway and cellular functions associated with this pathway by preventing or inhibiting Fas regulated apoptosis or growth and differentiation of cells. As used herein, the term "Fas-receptor mediated or modulated cellular finction" is to include any cellular response or function that has been linked to the binding of Fas or Fas/TNF receptor complex to its extracellular and/or intracellular ligand. Apoptotic cell death is one such response.

Methods of modulating cellular functions such as apoptotic cell death are provided herein. These methods comprise the steps of administering to the subject, such as an animal or human, an effective amount of a Pro-Yama, activated Yama, p20 and/or p11 Yama nucleic acid, antibody or protein. Alternatively, the method can be practiced using an inhibitory nucleic acid, antibody or protein. When the cellular function is augmentation of apoptotic cell death, an effective amount of a nucleic acid molecule coding for Pro-Yama or p20 and p11 Yama or their protein products can be administered to the subject. When the cellular function is inhibition or prevention of apoptotic cell death, an effective amount of a nucleic acid molecule coding for antisense Yama RNA, an anti-Yama antibody fragment, dominant inhibitory Yama, CrmA or their protein products are administered to the subject.

When practiced in vivo, the compositions and methods are particularly useful for modulating or regulating Fas receptor induced function in a subject or an individual suffering from or predisposed to suffer from receptor-related dysfunction or for maintaining T cell viability and function in a subject or an individual suffering from or predisposed to suffer from abnormal lymphocyte death, e.g., CD4+ T cell depletion associated with HIV infection. When the method is practiced in vivo in a human patient, it is unnecessary to provide the inducing agent since it is provided by the patient's immune system. When the method is practiced in vivo, the carrying vector, polypeptide, polypeptide equivalent, or expression vector can be added to a pharmaceutically acceptable carrier and systemically administered to the subject, such as a human patient or an animal such as a mouse, a guinea pig, a simian, a rabbit or a rat. Alternatively, it can be directly infused into the cell by microinjection or localized administration into a tumor. A fusion protein also can be constructed comprising the T-cell specific ligand for targeting to a T cell. Such T cell specific ligands include, but are not limited to anti-CD3, anti-CD4, anti-CD28 and anti-IL-1-receptor antibody.

This invention also is particularly useful to ward off lymphocyte death or immunosuppression in AIDS patients. By preventing or inhibiting apoptosis, not only is cell death prevented but functionality, e.g., immuno-proliferative capacity, is restored to the cell and a responsive immune system is retained or regained. Accordingly, the composi-

tions and methods of this invention are suitably combined with compositions and methods that prevent or inhibit HIV infectivity and replication.

The method also can be practiced ex vivo using a modification of the method described in Lum et al. (1993) *Bone Marrow Transplantation* 12:565–571 or a modification of the method described in U.S. Pat. No. 5,399,346. Generally, a sample of cells such as bone marrow cells or MLC can be removed from a subject or animal using methods well-known to those of skill in the art. An effective amount of the CrmA or a Yama nucleic acid molecule or expression vector is added to the cells and the cells are cultured under conditions that favor internalization of the nucleic acid by the cells. The transformed cells are then returned or reintroduced to the same subject or animal (autologous) or one of the same species (allogeneic) in an effective amount and in combination with appropriate pharmaceutical compositions and carriers.

Alternatively, fresh peripheral blood mononuclear cells (MNCS) isolated from the mammal or patient are separated from the red cells and neutrophils by Ficoll-Hypaque density gradient centrifugation. The MNCs are then washed, counted and cultured at approximately 1×10^6 cells/well in a 24 well tissue culture plates in AIM-V that consists of AIM-V (GIBCO) with 2mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml Fungizone and 25–1, 000U/ml of IL-2 (Cetus). The cells are cultured at 37° C. in a humidified incubator with 5% CO_2.

After the T cells have begun to proliferate, an appropriate insertion vector containing a CrmA or a Yama nucleic acid molecule is contacted with the cells to insert Yama nucleic acid into the proliferating cells. Multiple transfection of the cells may be necessary. The cells are maintained for an additional 2 to 7 days with fresh medium and under conditions to return the cells to exponential growth. Approximately 0.1 to $2.5–10^{10}$ T cells (or 80% of the total culture) are infused into the mammal or patient and the remaining cells can be cyropreserved for future infusions. A sample of the cells also can be removed for Southern analysis of insertion of the Yama nucleic acid molecule and its expression using northern analysis.

As used herein, the term "administering" for in vivo and ex vivo purposes means providing the subject with an effective amount of the nucleic acid molecule or polypeptide effective to modulate the Fas associated cellular function, e.g., to prevent, inhibit or augment apoptosis of the target cell. Methods of administering pharmaceutical compositions are well-known to those of skill in the art and include, but are not limited to, microinjection, intravenous or parenteral administration. The compositions are intended for topical, oral, or local administration as well as intravenously, subcutaneously, or intramuscularly. Administration can be effected continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well-known to those of skill in the art and will vary with the vector used for therapy, the polypeptide or protein used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. For example, the compositions can be administered prior to a subject already suffering from a disease or condition that is linked to apoptosis. In this situation, an effective "therapeutic amount" of the composition is administered to prevent or at least partially arrest apoptosis and the accompanying pathology such as immunosuppression in HIV infected individuals.

However, the compositions can be administered to subjects or individuals susceptible to or at risk of developing apoptosis-related disease to prevent pathological cell death. In one embodiment, the composition can be administered to a subject susceptible to HIV-related lymphocyte dysfunction to maintain lymphocyte cell function and viability. In these embodiments, a "prophylactically effective amount" of the composition is administered to maintain cellular viability and function at a level near to the pre-infection level.

It should be understood that by preventing or inhibiting unwanted cell death in a subject or individual, the compositions and methods of this invention also provide methods for treating, preventing or ameliorating the symptoms associated with a disease characterized by apoptosis of cells. Such diseases include but are not limited to AIDS, acute and chronic inflammatory disease, leukemia, myocardial infarction, stroke, traumatic brain injury, neural and muscular degenerative diseases, aging, tumor induced-cachexia and hair loss.

This invention also provides vector and protein compositions useful for the preparation of medicaments that can be used for preventing or inhibiting apoptosis, maintaining cellular function and viability in a suitable cell or for the treatment of a disease characterized by the unwanted death of target cells.

It also is intended that the compositions and methods of this invention be combined with other suitable compositions and therapies such as the use of CrmA, anti-idiotypic TCR antibodies, antagonists and Fas-receptor.

One aspect of this invention is based on Applicants' finding that the cowpox virus CrmA gene product is an exceptionally potent inhibitor of apoptosis induced by binding of a cell surface receptor to its ligand, e.g., TCR ligand, CTL, HIV, Fas ligand or TNF.

The only reported target for the CrmA protein is the cysteine protease interleukin-1β converting enzyme (ICE). In one embodiment that utilized TNF-and Fas- pathways; it is capable of blocking the cell death program even at pharmacological doses of the death stimulus. CrmA is a cowpox virus gene that encodes a protease inhibitor of the serpin family. The nucleic acid and corresponding amino acid sequences of CrmA have been reported (Pickup et al., *Proc. Natl. Acad. Sci.* (1986) 8:7698–7702) and are shown in FIG. **12**. However, Applicants have found that CrmA is an exceptionally potent inhibitor of apoptosis. Therefore, an important new function for CrmA is the prevention or inhibition of ligand-induced or cytokine-induced apoptosis. Further, the data suggest that a protease, either ICE or a related CrmA-inhibitable protein, is a component of the Fas- and TNF-induced cell death pathways. Thus, this invention provides: compositions and methods for preventing or inhibiting ligand-induced or cytokine-induced apoptosis; an assay for determining drugs or agents which facilitate or prevent or inhibit apoptosis; an assay for drugs to treat or ameliorate the symptoms associated with a disease or pathological conditions that occur as a result of apoptosis (such as AIDS); an assay for detecting the protease involved in the Fas- and TNF- induced cell death pathways, as well the proteases discovered using this method.

In one embodiment of this invention, the expression vector is to be specifically targeted to T cells. For these methods, it intended that the CrmA DNA be operatively linked to a promoter that is highly active in T cells. Such promoters include, but are not limited to: IFN-α; IL-2; IL-3; IL-4; IL-5; IL-9; IL-10; TFN-β; GM-CSF; CD4, CD8 and the IL-2 promoter.

Although the method is preferably practiced with the CrmA gene, it should be apparent to those of skill in the art that the polypeptide product of the CrmA gene and its biological equivalents are useful in the methods of this invention. The CrmA gene product is a 38 kDa protein and is known to be a specific inhibitor of IL-1β. It can be purified from natural sources as described in Ray, C. A. (1992) *Cell* 69:597–604 or produced recombinantly using the expression vectors described above in a host-vector system such as described in Pickup et al. (1986) supra, Ray et al. (1992) supra and Moss, B. ed. (1990) *Virology*, pp:2079–2112, Raven Press, N.Y. The protein is used in substantially pure form. By "substantially pure," it is meant that the protein is substantially free of other biochemical moieties with which it is normally associated in nature. The proteins also can be produced using the sequence provided in FIG. **5** and methods well-known to those of skill in the art.

Accordingly, this invention also provides a CrmA polypeptide, protein, a biological equivalent thereof and fusion proteins containing these, for use in the methods described herein. The polypeptides or proteins can be conjugated to targeting antibodies, such as anti-CD3 or anti-CD4 for targeted delivery to T cells.

This method can be practiced in vitro, ex vivo or in vivo. When the method is practiced in vitro, the expression vector, protein or polypeptide can be added to the cells in culture or added to a pharmaceutically acceptable carrier as defined below. In addition, the expression vector or CrmA DNA can be inserted into the target cell using well-known techniques such as transfection, electroporation or microinjection.

More specifically, the in vitro method comprises providing cell cultures or tissue cultures having either a cell surface receptor that mediates apoptosis such as a TCR, the TNF receptor or the Fas receptor. The cells are cultured under conditions (temperature, growth or culture medium and gas (CO_2)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. The cells are then exposed to preliminary conditions necessary for apoptosis, for example an effective amount of an inducing agent, e.g., a TCR ligand, HIV, SIV, TNF, or a Fas ligand such as an anti-Fas antibody is added to the culture. Anti-Fas antibodies and mitogens (ConA) are well-known to those of skill in the art. (Itoh, N. et al. (1991) *Cell* 66:233–243 and Yonehara et al. (1989) *J. Exp. Med.* (1989) 169:1747–1756). These cells are now "induced" to apoptosis. The cells are again cultured under suitable temperature and time conditions. In one embodiment, HIV or SIV is added to the culture. In other embodiments, a drug or agent to be tested is added in varying concentrations at a time that is simultaneous with, prior to, or after the inducing agent.

The CrmA nucleic acid or protein is then added to the culture in an effective amount and the cells are cultured under suitable temperature and time conditions to inhibit apoptosis. The CrmA nucleic acid or protein can be added prior to, simultaneously with, or after, the inducing agent. The cells are assayed for apoptotic activity using methods well-known to those of skill in the art and described herein. It is apparent to those of skill in the art that two separate cultures of cells must be treated and maintained as the test population. One is maintained without receiving an inducing agent to determine background release and the second without receiving the agent to be tested. The second population of cells acts as a control.

The use of the compositions and methods in vitro provides a powerful bioassay for screening for drugs that are agonists or antagonists of CrmA function in these cells. Thus, one can screen for drugs having similar or enhanced ability to prevent or inhibit apoptosis. It also is useful to assay for drugs having the ability to inhibit HIV infection and replication, since the $CD4^+$ cell will not die as a result of the concurrent viral infection. One of skill in the art can determine when the method has been successfully performed by noting the absence of apoptotic morphological changes or more simply, by the absence of cell death. The in vitro method further provides an assay to determine if the method of this invention is useful to treat a subject's pathological condition or disease that has been linked to apoptotic cell death in the individual.

In a separate embodiment, the T cell line designated CEM (ATCC) is obtained and used because it has been shown to undergo PCD upon infection with HIV. CEM cells are transfected by electroporation with the CrmA expression construct and vector alone as control. Clonal lines are derived and infected at various multiplicity of infection ratios with HIV. Cytopathic effect is assayed by microscopic observation and apoptosis quantitated following propidium iodine staining. Using the method described above, various agents can be tested for their ability to inhibit or prevent apoptosis.

EXPERIMENTAL

Assays to Assess Apoptosis

Apoptosis was assessed by several methods, described below.

Fluorescent DNA-Staining Dyes—Fluorescent DNA-staining dyes were used to reveal nuclear morphology. Transmission electron microscopy also was used. For propidium iodide staining, MCF7 cells were grown on 22 mm^2 No. 1 glass coverslips (Corning) placed in 35 mM wells of a 6-well culture dish (Costar). Following treatment with TNF, anti-Fas cycloheximide (CHX), or no treatment, medium was removed and the wells were rinsed twice with phosphate buffered saline (PBS), fixed in 100% methanol at −20° C. for 10 minutes, washed three times with PBS, and stained at room temperature for 10 minutes in a 100 μg/ml solution or propidium iodide (Sigma) made in PBS. The coverslips were then washed three times with PBS, blotted dry and mounted onto glass slides using Vectashield mounting medium for fluorescence (Vector Laboratories). BJAB cells were stained using acridine orange (Sigma) by preparing a wet mount of 30 μl of a cell suspension at a density of approximately 3×10^5 cell/ml mixed with 5 μl of a 100 μg/ml acridine orange solution made in PBS. Both propidium iodide-stained stained MCF7 and acridine orange-stained BJAB nuclei were visualized by fluorescence microscopy using a FITC range barrier filter cube. Laser-scanning confocal microscopy was performed using the Bio-Rad MRC 600 confocal microscope and digitized images obtained were artificially colorized.

For electron microscopy, cells were fixed and processed as per standard electron microscopy procedures.

Quantitative Apoptosis Assays—MCF7 cells or derived transfectants were plated at a concentration of 2.5×10^5 cells/well onto glass coverslips. Two days later, after the cells had adhered and spread, TNF or anti-Fas+CHK were added. TNF was added at a final concentration of 20 ng/ml, anti-Fas at 25 ng/ml, and CHX (Sigma) at 10 μg/ml. After 22 hours for the TNF treated samples or after 18 hours for the anti-Fas+CHX treated samples, cells were fixed, stained with propidium iodide and mounted as described- above. Apoptotic and non-apoptotic cells were quantitated based on

nuclear morphology using fluorescence microscopy and the percentage of non-apoptotic cells was calculated. A minimum of 100 cells was counted for each sample, and each experiment was done at least in duplicate. Since a small fraction of cells in any normally growing cell culture is undergoing apoptosis, spontaneous apoptosis in untreated or CHX along treated samples was also quantitated. The percentage of non-apoptotic cells in the TNF or anti-Fas+CHX treated samples was then normalized by correcting for the frequency of spontaneous apoptosis in the untreated or CHX alone samples, respectively.

BJAB cells were grown at 3×10^5 cells/ml and treated with anti-Fas antibody at a concentration of 250 ng/ml (unless indicated otherwise) for 18 hours after which an aliquot was stained with acridine orange as described above. Apoptotic cells and non-apoptotic cells were quantitated and normalized to untreated samples. Assays were done at least in duplicate.

MTT Conversion Assay—Secondary assays of cell death used the MTT conversion assay (as described in Opipari, A. W. et al. *J. Biol. Chem.* (1992) 267:12424–12427) and crystal violet staining as described in Tartaglia, L. A. et al. (1993) *Cell* 74:845–853.

DNA Fragmentation Assay—Assay of CTL-induced target cell DNA fragmentation was carried out as described previously in Duke (1992) infra, with modifications. Human PBMCs were prepared as described above. 1×10^7 BJAB cells were incubated with [methyl-^{3}H] thymidine pools. Cells were then plated as described above. After 4 hours, 100 μl of 95% ethanol was added to each well, the contents were mixed, and the plates were incubated for an additional hour. Ethanol addition caused the release of fragmented DNA while high molecular weight chromatin remained in the cells. The plates were then centrifuged, harvested, and analyzed as described above.

Cell Cultures—MCF7 cells, BJAB cells, and derived vector and CrmA stable transfectants along with the CrmA mutant-transfected stable lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, and nonessential amino acids and additionally supplemented with G418 sulfate (Life Technologies, Inc.) to 500 μg/ml for MCF7 transfectants and 3 mg/ml for BJAB transfectants.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)—20–50 ml of heparinized human whole blood was obtained from healthy donors and erythrocyte-depleted over Ficoll-Hypaque gradients. The resulting PBMCs were stimulated with PHA-P at 10 μg/ml for 3 days at 37° C. in RPMI supplemented with 10% fetal bovine serum prior to use in cytolysis and DNA fragmentation assays. CD4+ and CD8+ lymphocytes constituted approximately 40 and 30%, respectively, of the small lymphocyte pool in stimulated PBMCs as determined by flow cytometry. Subset depletion experiments confirmed that CTL activity at both 4- and 24-hour time points were mediated almost entirely by CD8+ T-lymphocytes. For measurement of natural killer (NK) cell activity, human PBMCs were used immediately after isolation without additional stimulation.

Plasmids, Transfections and Selection ofStably Transfected Lines—The CrmA gene as shown FIG. **12** and the mutant form were separately cloned into the pcDNA3 (Invitrogen) mammalian expression vector. The CrmA gene was obtained from Dr. David Pickup (Duke University) and used as a template in a PCR reaction using custom oligonucleotide primers with built-in restriction enzyme sites to amplify the entire coding sequence. The sequence of the primers are:

CrmA/5'/R1 (SEQ ID NO:8)
5' CAC CGG AAT TCC ACC ATG GAT ATC TTC AGG GAA

ATC G

CrmA/3'/XbaI (SEQ ID NO:9)
5' GCT CTA GAC TCG AGT TAA TTA GTT GTT GGA GAG

CAA TAT C

This PCR fragment was digested with EcoR1 and Xba1 restriction enzymes and subcloned into the pcDNA3 vector which had been similarly digested. Following transformation into competent XL-1Blue host *E. coli* cells (Stratagene), individual colonies were grown up, plasmid extracted and the presence of the CrmA gene confirmed by both restriction mapping and DNA sequence analysis.

The resulting expression construct or pcDNA3 itself (as the control) was introduced into both MCF7 and BJAB cells by electroporation. MCF7 cells were electroporated at 330 V, 960 TF in 0.4 cm cuvettes (BioRad), plated onto 100 mM dishes at varying dilutions and selected with G418 sulfate (Gibco-BRL) at a concentration of 500 μg/ml. After selection for three weeks, pooled populations from each transfection were prepared by trypsinizing dishes containing several hundred colonies. Additionally, clonal cells lines were derived by picking individual colonies from selected dishes. BJAB cells were electroporated at 220 V, 960 TF in 0.4 cm cuvettes (Bio-Rad) and selected in 3 mg/ml G418 sulfate. One day following transfection, a portion of the cell population was diluted at a concentration of 2500 cells/well in 96-well dishes from which clonal lines were obtained after G418 selection. The remainder of the cells were retained as the pooled population.

Cell Lines, TNF and Anti-Fas Antibody—The MCF7 cell line was a TNF-sensitive subclone obtained from Dr. David R. Spriggs (University of Wisconsin). MCF7 is a breast carcinoma epithelial cell line which expresses TNF receptor and is sensitive to TNF killing. The BJAB cell line was a gift of Dr. Fred Wang (Harvard). Recombinant TNF (specific activity 6.27×10^7 U/mg) was obtained from Genentech (South San Francisco, Calif.). Anti-Fas monoclonal antibody (clone CH-11, IgM) was obtained from Pan Vera (Madison, Wis.). The anti-PARP monoclonal antibody was clone C-2-10, which, as described previously (Lamarre et al. (1988) *Biochem. Biophys. Acta.* 950:147–160, recognizes an epitope near the N-terminal end of PARP, located between amino acids 216 and 375. The affinity-purified U1-70 kDa reactive antiserum was derived from human a autoimmune sera and was a kind gift from Dr. Antony Rosen (John Hopkins University, Baltimore, Md.).

Treatment with Anti-Fas Antibody or TNF and Preparation of Cell Lysates—MCF7 cells or derived transfectants were plated in 100-mm dishes at a concentration of 2×10^6 cells/dish. On the second day, cells were treated with TNF at 40 ng/ml for the indicated time periods. Following a PBS rinse, cells were harvested by scraping into 15 ml of PBS plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml aprotinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepstatin), recovered by centrifugation, and lysed in 2.5 ml of sample buffer (50 mM Tris-HCl, pH 6.8,6 M urea, 6% 2-mercaptoethanol, 3% SDS, and 0.003% bromphenol blue). In cases where nonadherent cells were present in the culture medium (e.g. at later time points), floating cells were also harvested by centrifugation and combined with the

adherent cell pellet before lysis in sample buffer.BJAB cells or derived transfectants were aliquoted at a concentration of 5×10^5/ml into six-well dishes, with 4 ml in each well. The following day, cells were treated with anti-Fas antibody (250 ng/ml) for the indicated time periods, harvested by centrifugation, washed once with PBS plus protease inhibitors, and lysed in 2 ml of sample buffer.

Western Blotting

Western Blotting—Immunoblotting to assess the state of U1-70 kDa was carried out similarly as described in Casciola-Rosen, L. A., et al., (1994) *J. Biol. Chem.* 269:30757–30760. In brief, cell lysates (20 μl) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher and Schuell) by electroblotting. Blots were blocked at room temperature for 1 hour using blocking buffer (10 mM Tris (pH 7.6),150 mM NaCl, 0.5% Nonidet P-40, 3% bovine serum albumin). The U1-70 kDa affinity-purified antiserum was used at a 1:5000 dilution in blocking buffer and was incubated for 1 hour at room temperature. The secondary reagent, a biotinylated goat anti-human IgG antibody (Southern Biotech), was used at a dilution of 1:6700 in blocking buffer and was incubated for 50 minutes at room temperature. The tertiary reagent, a streptavidin-horseradish peroxidase conjugate (Southern Biotech), was used at a dilution of 1:10,000 in blocking buffer and was incubated at 30 minutes at room temperature. Visualization of signal was by electrochemiluminescence (Amersham Corp.).

Cytotoxicity Assays

Cytotoxicity Assays—PHA-facilitated allegeneic CTL assays using PHA—stimulated human PBMCs were carried out as described in Grabstein, K. (1980) in *Selected Methods in Cellular Immun.* (Mishell and Shiigi, eds.) pp. 125–137, W. H. Freeman and Co., New York, N.Y., with minor modifications. Briefly, following PBMC stimulation, $2–10^7$ BJAB target cells were incubated with 200 μCi of $(Na)_2{}^{51}CrO_4$ (ICN) in a 400-μl total volume of Hanks' balanced salt solution, 0.2% bovine serum albumin for 2 hours at 37° C. Target cells were then washed and plated in round-bottom 96-well plates at 10,000 cells/well. Human PBMCs were plated at various killer to target ratios, ranging from 0.25:1 to 32:1, and PHA-P was added to a final concentration of 10 μg/ml and a total volume of 200 μl. In some experiments, EGTA and $MgCl_2$ were added at 10 and 4 mM, respectively, to clamp intracellular calcium levels. At 4 and 24 hour time points the plates were centrigued, and 30-μl aliquots were harvested and spotted onto glass fiber filters. Samples were analyzed using a β scintillation counter. Specific cytotoxicity (%) was calculated as ((sample cpm—spontaneous release cpm)/(total release cpm—spontaneous release cpm))×100. Background release of chromium was typically 5–15% at the 4-hour time point and 20–30% at the 24-hour time point. The addition of EGTA/$MgCl_2$ did not by itself affect cell viability as measured by both background chromium release and morphologic examination of cells at both time points. There was no significant difference in background ^{51}Cr release between vector and CrmA-transfected lines. NK cell cytotoxicity assays were carried out as described above except that the cells were not stimulated and there was no PHA present during the assay.

RNA Isolation and Northern Analvsis

RNA Isolation and Northern Analysis of CrmA—RNA isolation and northern analysis were carried out as described in Dixit et al. (1990) *J. Biol Chem.* 265:2973–2978. PCR (Perkin-Elmer) was used to generate a probe spanning the coding region of the CrmA gene as described above. β-actin cDNA probe was purchased from Clontech (Palo Alto, Calif.) and the hybridization signal was visualized as a digitized image on a Molecular Dynamics Phosphorimager.

EXPERIMENT NO. 1

Induction ofApoptosis by TNF and anti-Fas—A subclone of the MCF7 breast carcinoma epithelial cell line which expressed TNF receptor and was sensitive to TNF killing was chosen for these studies. This cell line is characterized in Spriggs et al. (1988) *J. Clinc. Invest.*, 81:455–460. Further analysis revealed that Fas was also expressed on these cells and that crosslinking with an anti-Fas monoclonal antibody in the concomitant presence of the protein synthesis inhibitor cycloheximide induced cell death.

Cycloheximide aline for the duration of the assay did not induce cell death beyond the negligible frequency of spontaneous apoptosis which is observed in any untreated cell culture. Anti-Fas alone was not cytotoxic, but this is not surprising, since induction of cell death in non-lymphoid cells by Fas activation has been reported to require the concomitant presence of either transcriptional or translational inhibitors. See Itoh et al. (1991) *Cell* 66:233–243.

A B-cell lymphoma cell line (BJAB) also was examined. It expresses a high level of Fas and was killed by the addition of anti-Fas antibody in the absence of a protein synthesis inhibitor.

Cell death can occur by two biochemically and morphologically distinct processes: apoptosis and necrosis. In these studies, cell death was first confirmed to be the result of TNF or anti-Fas induced apoptosis, not necrosis. Although various markers of apoptosis have been reported, the phenomenon is preferably defined at the morphological level and is characterized by chromatin condensation and margination along the inner nuclear membrane, cytoplasmic condensation and membrane blebbing without disintegration of the cellular membrane. See Duvall et al. (1986) *Immunol. Today* 7:115–119. Conversely necrosis is defined by cytoplasmic swelling and lysis of the cell membrane and, importantly, does not exhibit the chromatin margination characteristic of apoptosis. DNA laddering, representative of cleavage at intemucleosomal intervals, is seen in some but not all forms of apoptosis, further emphasizing the importance of morphological criteria in defining apoptosis. See Barres et al. (1992) *Cell* 70:31–46. Nuclear morphology of cells dying in response to TNF or anti-Fas antibody was examined following staining with the DNA-binding dyes propidian iodine (MCF7 cells) and acridine orange (BJAB cells). Fluorescence microscopy laser scanning confocal microscopy demonstrated marked changes in nuclear morphology in the MCF7 cells in response to either TNF or anti-Fas-CHX and in the BJAB cells in response to anti-Fas. Chromatin condensation was clearly visible by immunofluorescence microscopy in both cell lines and formed the basis for the later assays of apoptosis in transfected cell lines. Confocal microscopy confirmed margination along the inner nuclear membrane. These morphological criteria of apoptotic cell death were further confirmed by transmission electron microscopy. The MCF7 cells clearly demonstrated chromatin condensation and margination along the inner nuclear membrane, cytoplasmic condensation and increased membrane blebbing in response to either TNF or anti-Fas+ CH. BJAB cells treated with anti-Fas antibody demonstrated chromatin margination and cellular shrinkage typical of

apoptosis in lymphoid cells. Thus, both TNF and Fas induced genuine apoptotic cell death in these cell lines.

EXPERIMENT NO. 2

Generation of CrmA-mutant plasmidsfor eukaryotic, bacterial and in vitro expression—A four-primer PCR-based method (as described in Higuchi et al. (1988) *Nucleic Acids Res.* 16:7351–7367) was employed to convert codon 291 in the CrmA gene from Thr to Arg. The wild-type sequence of the cDNA and protein are shown in FIG. **8**. Initially, two independent PCR reactions were performed using the plasmid pcDNA3/CrmA (as described in Tewari and Dixit (1995) *J. Biol. Chem.* 270:3255–3260) and described above) as template. One reaction consisted of an upstream primer (Primer A) corresponding to nucleotides 682–711 of the CrmA coding sequence (with nucleotide 1 representing the first nucleotide of the initiator methionine codon), and a downstream mutagenic primer (Primer M2) complementary to nucleotides 853–896. Primer M2 contained a G to A transition which eliminated a Pst 1 site and base changes which altered codon 291 to encode Arg instead of Thr and, additionally, introduced a diagnostic Nru 1 site. The second PCR reaction used an upstream sense mutagenic primer (Primer M1) complementary to primer M2 and a downstream primer (Primer B) complementary to the last 26 nucleotides of the CrmA coding region with custom Xba 1 and Xho 1 sites. The PCR products were gel purified, combined, denatured by boiling and annealed by slow cooling to room temperature. Following a 10 minute extension reaction, PCR was carried out using the flanking primers A and B. The amplification product was digested with Cla 1 (cuts at nucleotide 692 in the CrmA coding sequence) and Xba 1 and cloned into pcDNA3/CrmA which had been similarly digested. The mutation was verified by DNA sequencing, as were all segments derived by PCR amplification. This recombinant plasmid was designated pcDNA3/CrmA-mutant.

The sequences of oligonucleotide primers were as follows: Primer A. (SEQ ID NO: 10) 5' GCT ATG TTT ATC GAT GTG CAC ATT CCC AAG; Primer M2 (SEQ ID NO: 11): 5' GCA CAA GTT GCT GCG GCT GCT TCG CGA TAC TCT TCA TTG ACA TC;

Primer B (SEQ ID NO: 12), 5' GCT CTA GAC TCG AGT TAA TTA GTT GTT GGA GAG CAA TAT C;

Primer M1 (SEQ ID NO: 13), 5' GAT GTC AAT GAA GAG TAT CGC GAA GCA GCC GCA GCA ACT TGT GC.

For purposes of in vitro transcription and translation, the native CrmA gene and its mutant version were digested with Nco 1 and Xho 1 and ligated into a plasmid based on pTM1 (Moss et al. (1990) *Nature* 348:91–92) that encoded an in-frame N-terminal Met-His_6 tag to facilitate purification. The coding sequence started with the initiator methionine, followed by six histidines, a serine and then the entire coding region of CrmA or the mutant.

For expression in *E. coli*, the native and mutant CrmA genes in pTM1 were digested with Nco 1 and Xho 1 and ligated into a derivative of the isopropyl-1-thio-J-D-galactopyranoside (IPTG)-inducible plasmid pFLAG (IBI) that contained the same His_6 fusion tag. Additionally, the CrmA gene from pcDNA3/CrmA (Tewari and Dixit (1995) supra was subcloned into the Nco 1/Xho 1 digested pGSTag bacterial expression vector (provided by Dr. Holly Dressler, a Massachusetts General Hospital and described in Ron and Dressler (1992) *BioTechniques* 13:866–869) generating a chimeric glutathione-S-transferase (GST)-CrmA open reading frame.

Preparation of GST-CrmA fusion protein and generation of rabbit polyclonal antiserum—Antibodies were raised against recombinant CrmA fusion proteins. Initial immunization was with the 6xHis-tagged CrmA recombinant protein and subsequent immunizations were with a GST-CrmA fusion protein produced as described previously (Hu et al. (1994) *J. Biol. Chem.* 269:30069–30072). Briefly, the BL21pLyS *E. coli* strain was transformed with pGSTag-CrmA plasmid and production of fusion protein induced in culture by the addition of IPTG to 50 μM. Following a 1.5 hour incubation at 25° C., the cells were recover ed by centrifugation, resuspended in lysis buffer (20 mM Tris pH 8.0, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml pepstatin and 0.1% Triton X-100), sonicated, clarified by centrifugation, and absorbed to glutathione-agarose beads (Sigma). Soluble GST-CrmA fusion protein was eluted by incubating with 5 mM free glutathione (Sigma). Typical yield of fusion protein was 1 mg per liter of bacterial culture. Immunization of rabbits and screening of antisera was as previously described (O'Rourke et al. (1992) *J. Biol. Chem.* 267:24921–24924).

Expression andpurification of recombinant 6xHis-tagged CrmA proteins from *E. coli—E. coli* strain TG1 transformed with either the 6xHisCrmA or 6xHisCrmA-mutant construct was induced with IPTG for 3 hours, harvested and the cells lysed by sonication was pelleted by centrifugation. The supernatant contained soluble CrmA was filtered through a 0.22 μm filter, loaded onto a 2 ml Ni-NTA column (Qiagen), and washed with 50 mM Tris pH 8.0 containing 0.5 M NaCl. CrmA was eluted with 50 mM Tris, 50 mM imidazole pH 8.0, containing 0.1 M NaCl. This material was diluted with 9 volumes of 20 mM Hepes pH 7.4 containing 2 mM dithiothreitol and applied to a 2 ml column of DEAE Sepharose. This column was developed with a linear gradient of 0–1 M NaCl in 20 mM Herpes buffer, pH 7.4, and CrmA eluted at approximately 0.4 M NaCl to give a yield of 4 mg protein from six liters of culture. The material was greater than 95% pure as estimated by Coomassie blue staining and was stored at −70° C. until use. In all experiments using this material, the CrmA was treated with 2 mM DTT for 5 minutes immediately before use. This resulted in CrmA with the highest inhibitory activity.

In vitro assay of ICE inhibition by recombinant CrmA or CrmA-mutant protein—To assay for inhibition, 44 ng of purified ICE was activated with 10 mM DTT for five minutes at room temperature, then incubated at 37° C. with various amounts of purified CrmA or CrmA-mutant protein in a total volume of 95 μl of reaction buffer: 20 mM Hepes buffer, pH 7.4, containing 100 mM NaCl, 0.5% NP40, and 10 mM DTT. After 15 minutes, 5 μl of a 10 mM stock in DMSO of Boc-Ala-Ala-Pro-Asp-p-(SEQ ID NO: 14)nitroanilide was added to determine the residual ICE activity by observing the release of p-nitroaniline at 410 nm using a Molecular Devices V_{max} plate reader operating in the kinetic mode. The data were expressed as the reaction velocity in the presence of inhibitor (v_i) divided by the velocity in its absence (v_o), which represents residual ICE activity. The data represent the mean and standard deviations of values derived from two independent experiments. Purified recombinant human ICE was supplied by Nancy Thomberry (Merck).

In vitro transcription/translation of CrmA and CrmA-mutant—Coupled transcription/translation was performed using the TNT® kit from Promega according to the manufacturer's recommendations. Briefly, 0.5 μg of plasmid DNA was incubated for one hour at 31° C. in a total volume of 50

μl containing the kit reagents and 20 μCi of translation grade [^{35}S]Met. Once translated, the reaction mix was either used immediately or stored at −20° C. until needed.

Gel-Shift assays to detect complexformation between ICE and CrmA or CrmA-mutant—Serpin reactions with target proteases can be analyzed by gel-shift analysis using purified proteinases and [^{35}S]Met labelled serpins produced by in vitro translation, as described in Komiyama et al. (1994) "*Techniques in Protein Chemistry*" Acad. Press, San Diego, Calif., pp. 305–312 and Komiyama et al. (1994) *J. Biol. Chem.* 269:19331–19337. In-vitro transcribed and translated CrmA or CrmA-mutant was diluted with an equal volume of 50 mM Hepes buffer pH 7.4 containing 100 mM NaCl, 10% sucrose, and 0.1% CHAPS. 10 μl of the diluted lysates were incubated with 10 μl of consecutive three-fold dilutions of ICE in the same buffer containing 10 mM DTT for 30 minutes at 37° C. Samples were then resolved by native gel electrophoresis and visualized using a Molecular Devices Phosphorimager.

Transverse Urea Gradient PAGE—In vitro translated CrmA or CrmA—mutant protein was subjected to electrophoresis in transverse urea gradient (TUG) polyacrylamide gels (0 to 8 M) as previously described in Goldenberg (1989) "Protein Structure: A Practical Approach" IRL Press, N.Y. pp. 225–250 and Mast et al. (1991) *Biochem.* 30:1723–1730. The gels were dried and analyzed using a Molecular Devices Phosphorimager.

Stable transfection of BJAB and MCF7 cells—MCF7 or BJAB cells were electroplated with pcDNA3/CrmA-mutant plasmid and stable clonal cell lines generated as previously described (Tewari and Dixit (1995) supra).

MCF7 cells, BJAB cells and derived vector and CrmA stable transfectants, along with CrmA-mutant transfected stable lines generated in this study were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, nonessential amino acids and additionally supplemented with G418 sulfate (Gibco-BRL/Life Technologies, Inc.) to 500 μg/ml for MCF7 transfectants and 3 mg/ml for BJAB transfectants.

CrmA Blocks TNF- and anti-Fas-Induced Apoptosis—To determine whether CrmA can function to inhibit cytokine-induced apoptosis, MCF7 and BJAB cell lines were transfected with either the expression vector pcDNA3 by itself or as a CrmA expression construct. Expression of the CrmA gene was confirmed by northern analysis. Stable transfectants were generated by neomycin selection, and pooled populations of neomycin-resistant cells were assayed for CrmA expression. These pooled populations were analyzed for their sensitivity to TNF- and anti-Fas-induced apoptosis by direct quantitation of apoptotic cells based on nuclear morphology following staining with DNA-binding dyes and visualization by fluorescence microscopy. Dramatic resistance was seen with either TNF or Fas in both cell lines. This was remarkable, given that in the pooled population of neomycin-resistant cells transfected with CrmA, a significant fraction of cells were likely not expressing CrmA due to, among other reasons, nonproductive integration of the expression construct into genomic DNA.

In addition to the pools, clonal lines were derived from both MCF7 and BJAB transfectants and challenged by activation of the TNF and Fas death pathways. In the MCF-7 cell line, vector clones were uniformly sensitive to apoptosis induced by either TNF or anti-Fas+CHX, whereas among the transfected clones, those which expressed detectable CrmA were totally resistant to apoptosis (FIG. **3**A). Indeed, lines expressing the highest levels of CrmA were totally resistant to apoptosis (FIG. **3**A) and showed no morphologic cytopathic effects (FIG. **3**B), demonstrating the CrmA can completely block the TNF- and Fas-mediated death pathways. Similarly, among BJAB transfected clones, the CrmA expressing the lines were markedly resistant to anti-Fas induced apoptosis whereas the vector clones were universally sensitive (FIG. **4**A). Importantly, in both MCF7 and BJAB transfectants, those clones expressing the highest levels of CrmA were the most resistant while those clones expressing little or undetectable levels were the most sensitive (FIG. **3**A and FIG. **4**A). Although direct visual quantitation of apoptotic nuclei is the preferable measure of apoptosis, comparable results were obtained when either an MTT-conversion based death assay (FIG. **3**A insert and FIG. **4**A inset) or crystal violet staining (FIG. **3**C) was employed to assess cell survival.

The dose of death stimulus was increased to determine if protection conferred by CrmA from cytokine-induced apoptosis could be attenuated. Remarkably, CrmA afforded comparably high levels of protection from anti-Fas-induced apoptosis in response to doses of antibody 250 times greater than those needed to kill greater than 95% of the vector transfected cells (FIG. **4**B). Similar results were obtained when the dose of TNF was similarly varied for the MCF7 transfectants, implying that CrmA is functioning as an exceptionally potent inhibitor of cell death at a presumably critical step in the death pathway.

These results describe an important new function for CrmA—the blockade of TNF- and Fas-mediated apoptosis. Given the importance of both TNF and Fas in the host anti-viral response, it is likely that this function of CrmA is important for productive viral infection in vivo. CrmA represents yet another example of viral economy in which two important functions, namely the inhibition of IL-1β production and the prevention of apoptosis, are embedded in one protein.

Additionally, this data have implications for the unification of death pathways in general. First, the fact that CrmA blocks both TNF- and Fas-mediated apoptosis, especially in the MCF7 cells that possess both receptors, suggests that they signal death through a biochemically common pathway. This hypothesis is supported by the finding that the cytoplasmic regions of both these receptors encompass a region of homology which has been defined by mutational analysis as a "death domain" and which presumably interacts with a common set of signal transduction molecules. Further, it is not apparent that CrmA blocks cell death triggered by two very different stimuli: growth factor withdrawal in neuronal cultures and, activation of cytokine receptors. It is of note that apoptosis in these two systems has been suggested to occur through biochemically distinct pathways, in that apoptosis in the former system is dependent on new protein synthesis and death is blocked by cycloheximide, whereas in contrast TNF- and Fas-mediated cytotoxicity is independent of new protein synthesis and is, in fact, enhanced by cycloheximide. Thus, at some point, the death pathway in both systems converges upon a CrmA-inhibitable step, likely the activation of a protease. Applicant has identified this protease as Pro-Yama that is activated by PARP.

EXPERIMENT NO. 3

To determine whether CrmA can function to inhibit the lethal cascade induced upon interaction of the CTL with its target, BJAB cells (a Fas-expressing human B-cell line), were stably transfected with either vector control-, CrmA, or inactive point mutant CrmA expression constructs as target cells in a 24-hour PHA-facilitated allogeneic CTL-mediated

cytolysis assay based on ^{51}Cr release from target cells. ^{51}Cr release was examined after 24 hours because it was anticipated that both CA^{2+}-dependent and CA^{2+}-independent cytotoxicity would occur at this time point. Two vector-transfected clones (V1 and V4), two CrmA-transfected clones (CrmA 2 and CrmA 3), and two mutant CrmA-expressing clones (mutant CrmA 12 and mutant CrmA 17) were selected that have been previously described and characterized above. (See Vaux, D. et al. (1994) *Cell* 76:777–779; Gagliardini, V. et al. (1994) *Science*, 263: 826–828; Enari, M. et al. (1995) *Nature*, 375: 78–81; Los, M. et al. (1995) *Nature*, 375: 81–83; Quan, L. T. et al. (1995) *J. Biol. Chem.*, 270: 10377–10379; Grabstein et al. (1980), supra and Duke, R. C. and Cohen, J. J. (1992) in *Current Protocols in Immun.* (Coligan, J. E. et al. eds.) Vol. 1, pp. 3.17.11–13.17.16, John Wiley and Sons, New York, N.Y.). Mutant CrmA carries a Thr→Arg point mutation at codon 291, a critical site in the reactive site loop of CrmA. This mutation abolishes protease inhibitory capacity without significantly altering tertiary structure, as measured by transverse urea gradient gel electrophoresis as described herein. As shown herein, this mutation has also been shown to abolish the ability of CrmA to inhibit tumor necrosis factor- and Fas-induced cell death.

MCF7 cells, BJAB cells, and derived vector and CrmA stable transfectants along with the CrmA mutant-transfected stable lines were maintained as noted above. To determine cell killing and whether apoptosis had occured, the following assays were utilized.

PHA-facilitated allegeneic CTL assays using PHA-stimulated human PBMCs were carried out as described previously in Grabstein, K. (1980) supra, with minor modifications. This assay is described above.

NK cell cytotoxicity assays were carried out as described above except that the cells were not stimulated and there was no PHA present during the assay.

When examined for susceptibility to CTL-mediated cytolysis, the parental BJAB cell line was killed effectively, as were vector transfected cells (FIG. **13**A, clones V1 and V4), in a dose-dependent fashion over a range of killer:target cell ratios. Lines expressing CrmA, however, were significantly protected from CTL-mediated killing (FIG. **13**A, clones CrmA 2 and CrmA 3). When spontaneous background release of ^{51}Cr from BJAB lines incubated for 24 hours without the addition of CTLs was assessed, there was no significant difference between vector and CrmA lines. Thus, the difference in susceptibility to killing between vector and CrmA-expressing lines in the CTL-mediated cytolysis assays is not simply due to a decreased intrinsic propensity of the CrmA lines to undergo spontaneous lysis.

To determine whether the ability of CrmA to inhibit CTL-mediated cytolysis required its protease-inhibitory capacity, cell lines were examined that expressed comparable levels of the point mutant of CrmA, that, as mentioned above, lacks protease inhibitory activity. When tested in the same cytotoxicity assay, both lines expressing mutant CrmA were as sensitive to CTL-mediated cytolysis as a vector transfectant, whereas wild-type CrmA was clearly protective (FIG. **13**B).

Because killing by CTLs is the result of activation of both granzyme and Fas pathways, it was of interest to determine whether one or both of these was modulated by CrmA, since previous studies indicated that CrmA could potentially inhibit both pathways. To investigate this, Ca^{2+} dependence of granzyme-mediated killing and the Ca^{+} independence of the Fas pathway was utilized. The calcium chelator, EGTA, can be used to block granule exocytosis, since calcium is necessary for granzyme release from the CTL. Thus, one can selectively assess Ca^{2+}-independent killing. In the absence of EGTA, CrmA was protective compared with vector controls, but some residual cytotoxicity was present (FIG. **14**A). In the presence of EGTA, however, CrmA was totally protective (FIG. **14**B), indicating that CrmA can completely block the Ca^{2+}-independent component of CTL killing.

Studies of Granzyme B-knockout mice have suggested that ^{51}Cr release in the first 4 hours after CTL-target interaction is likely to involve the granzyme-based pathway only. The experiments reported in the cytolysis assay above were employed in place of the 24-hour assay. In this 4-hour assay, there were no significant differences in CTL sensitivity of vector-transfected and CrmA-transfected target cells (FIG. **15**A). Furthermore, EGTA abolished all cytotoxicity in the 4-hour assay (FIG. **15**B), confirming that the cytolysis at this time point was entirely due to the Ca^{2+}-dependent mechanism and indicating that CrmA therefore does not afford protection from the Ca^{2+}-dependent pathway. CrmA also did not confer any protection from death induced by NIK cells. This result is consistent with the hypothesis that CrmA does not block the Ca^{2+}-dependent component of cytotoxicity, since NK-mediated cytolysis appears to be entirely accounted for by the degranulation pathway.

Whereas the 4-hour ^{51}Cr release assay measured only Ca^{2+}-dependent cytolysis, assessing DNA fragmentation was a more sensitive measure of total apoptosis at this early time point, as it precedes membrane lysis in the case of Fas-mediated mediated apoptosis. When both vector control and CrmA-transfected lines were examined for induction of DNA fragmentation by CTLS, CrmA-expressing lines showed significantly less DNA fragmentation (FIG. **16**), consistent with protection from early DNA fragmentation induced by Fas ligation.

EXPERIMENT NO. 4

Cloning of a cDNA encoding Yama—The cDNA clone (b4HB3MA-COT8-HAP-Ft280 5') corresponding to EST T10341 was kindly provided by M. Bento Soares (Columbia University) and used to screen a random-primed cDNA library constructed from human umbilical vein endothelial cells treated with TNF and cycloheximide. Double-stranded DNA sequencing revealed an open reading frame, designated Yama, of 277 amino acids.

Expression and purification of 6xHis-tagged Yama—A 2.3 kb Nco 1/Bam Hi fragment from the Yama cDNA described above was ligated into a vector (pTM1) that contained an N-terminal His_6 fusion to facilitate purification. This construct contained the 6xHis tag followed by the complete coding region of Yama along with 1800 bp of 3' untranslated DNA. Coupled transcription/translation was performed with the TNT® kit (Promega) according to the manufacturer's recommendations with modifications. Briefly, 4μg of plasmid DNA was incubated for 1 hour at 31° C. in a total volume of 400 μl containing the kit reaction mixes and 160 μCi of translation grade [^{35}S]Met. The translation reaction was diluted 1:20 with 20 mM Hepes buffer, pH 7.4, loaded onto an equilibrated 500 μl DEAE sepharose (Pharmacia) column, then washed with 8 ml of Hepes buffer. The column was eluted with 5 ml of 20 mM Hepes, 0.5M NaCl. This eluate was loaded onto a 300 μl Ni-NTA column (Qiagen), then washed with 5 ml of Reaction Buffer (50 mM Hepes pH 7.4, 0.1 M NaCl, 0.1% CHAPS, and 10% sucrose). The protein was eluted with 5×400 μl fractions of Reaction Buffer with 50 mM imidazole.

Activation of Yama and in vitro reconstitution experiments—Purified Yama (20 μl) was activated by incubating at 37° C. for 4 hours with 1.5 pmol ICE in Reaction Buffer supplemented with DTT (10 mM) in a total volume of 25 μl, after which 30 μl of Reaction Buffer was added and the reaction incubated at 37° C. for an additional 15 minutes. Following activation, 30 μl of either control Reaction Buffer or Reaction Buffer containing 270 pmol recombinant 6xHis-CrmA or 270 pmol recombinant 6xHisCrmA-mutant was added and allowed to incubate at 37° C. for 30 minutes. The recombinant proteins as well as the control buffer had been pre-incubated with DTT (2mM) to pre-activate CrmA. Following the 30 minute incubation, 2 μl (0.586 μg) purified PARP was added and the DTT concentration raised to 10 mM, following which the reaction was allowed to proceed for 2 hours at 37° C. The PARP alone control reaction was carried out under identical conditions, except that no Yama, ICE, or CrmA proteins were added during the procedure. The ICE+PARP reaction was carried out identically as well, except that no Yama or CrmA proteins were added during the procedure.

Following the incubation with PARP, one-fifth of each reaction sample was analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10 as described later. Additionally, an equivalent amount of each sample was resolved by SDS-PAGE and analyzed using a Molecular Devices Phosphorimager to assess the state of radiolabeled Yama present in the reaction. Purified ICE was a gift of Nancy Thomberry (Merck). PARP was purified as has been described in Zahradka and Ebisuzaki (1984) *Eur. J. Biochem.* 142:503–509.

CrmA immunoprecipitation to detect complex formation with Yama—Reactions were assembled by combining 20 μl of [^{35}S]-Met-labeled Pro-Yama or ICE-activated Yama with either 358 pmol native CrmA or 358 pmol mutant CrmA protein. Each reaction was diluted to a final volume of 80 μl in Reaction Buffer. Complex formation was allowed to occur at 37° C. for 30 minutes. Ten (10) μl of each reaction was resolved by SDS-PAGE and subjected to phosphorimaging to visualize the radiolabeled Pro-Yama or activated Yama. Thirty-five (35) μl of each reaction was diluted to 1 ml in PBS-TDS (as described in O'Rourke et al. (1992) *J. Biol. Chem.* 267:24921–24924) and immunoprecipitated using 25 μl of the rabbit polyclonal CrmA antiserum (described later in this section). Immunoprecipitation was carried out as described in O'Rourke et al. (1992) supra and precipitates were resolved by SDS-PAGE and subjected to phosphorimaging analysis to detect the presence of radiolabeled Pro-Yama or activated Yama. Coomassie blue staining of the gel revealed that equivalent amounts of native CrmA and mutant CrmA were precipitated by the CrmA antiserum.

Treatment with Anti-Fas or TNF and Preparation of Cell Lysates for PARP Analysis—MCF7 cells or derived transfectants were plated in 100 mM dishes at a concentration of 2×10^6 cells per dish. On the second day, cells were treated with TNF at 40 ng/ml for the indicated time periods. Following a PBS rinse, cells were harvested by scraping into 15 ml PBS+Protease Inhibitors (1 mM PMSF, 0.5 mg/ml aprotinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepstatin), recovered by centrifugation and lysed in 2.5 ml Sample Buffer (50 mM Tris-HCl, pH 6.8. 6 M urea, 6% 2-mercaptoethanol, 3% SDS and 0.003% bromophenol blue). In cases where nonadherent cells were present in the culture medium (e.g., at later time points), floating cells were also harvested by centrifugation and combined with the adherent cell pellet before lysis in Sample Buffer.

BJAB cells or derived transfectants were aliquoted at a concentration of 5×10^5/ml into six-well dishes, with 4 ml in each well. The following day, cells were treated with anti-Fas antibody (250 ng/ml) for the indicated time periods, harvested by centrifugation, washed once with PBS+ Protease Inhibitors and lysed in 2 ml Sample Buffer.

For detection of CrmA, whole cell lysates (2×10^5 cells per lane) were resolved by SDS-PAGE, transferred to nitrocellulose and processed as previously described herein. The anti-GST-CrmA rabbit antiserum was used at a dilution of 1:1,000 and a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham Life Sciences) used at a 1:15,000 dilution. Visualization of signal was by ECL (Amersham).

Immunoblotting of lysates for PARP was carried out as described in Desnoyers et al. (1994) *Anal. Biochem.* 218:470–473. The anti-PARP mouse monoclonal antibody was used at a dilution of 1:10,000 and the secondary antibody, an anti-mouse Ig labeled with horseradish peroxidase, was used at a dilution of 1:1,000. Visualization of signal was also by ECL.

A human umbilical vein endothelial cell library was screened from which a cDNA encoding an open reading frame of 277 amino acids, designated Yama was cloned. Yama is homologous to the Ced-3/ICE family of proteins. Yama was then assayed for protease activity and to determine if it fulfilled the requirements predicted for a death protease. The amino acid sequence suggested that it would be an Asp-specific cysteine protease, as the residues thought to be important for Asp-specificity in ICE are conserved in Yama. Yama was expressed in vitro as a fusion to a GxHis purification tag at the N-terminus and isolated by ion exchange and nickel chelate affinity chromatography to determine if it possessed proteolytic activity capable of cleaving PARP. The full-length, p32 form of purified Yama had no proteolytic activity against PARP (FIG. **2**A, Top, Lane 3) and is thus designated Pro-Yama. Purified ICE was able to cleave Pro-Yama to yield two major products (FIG. **2**A, Bottom, Lane 4; putative p20 and p11 subunits are indicated by open arrows) and, more importantly, that Yama activated in this manner acquired proteolytic activity and cleaved PARP to the 85 kDa apoptotic form (FIG. **2**A, Top, Lane 4). Purified ICE did not cleave PARP (FIG. **2**A, Top, Lane 2), confirming the earlier results of Lazebnik et al. (1994) *Nature* 371:346–347, and excluding the possibility that PARP cleavage was mediated by the added ICE. The next investigation concerned whether PARP cleavage occurred in these cells death systems and whether the cleavage product was analogous to that observed in the in vitro experiments. Activation of either Fas (in BJAB lymphoma cells) or TNF receptors (in MCF7 breast carcinoma cells) induced PARP cleavage to the signature 85 kDa form (FIG. **2**B), and this product co-migrated with the PARP cleavage fragment generated by purified Yama (FIG. **2**A, Top, Lanes 4 and 5). Thus, Yama is a protease which cleaves PARP to the signature 85 kDa apoptotic fragment.

Since the mammalian cell death protease is expected to be susceptible to inhibition by CrmA, whether Yama was CrmA-inhibitable was then investigated. To address this question definitively, purified proteins were used to reconstitute the PARP cleavage reaction in vitro such that purified recombinant CrmA protein could be added. To serve as a control, a point mutant of CrmA was constructed. CrmA-mutant carries a single amino acid substitution of Arg for Thr at amino acid 291 (FIG. **3**A). Both CrmA and CrmA-mutant proteins were expressed as 6xHis-tag fusion proteins in *E. coli* and purified by nickel chelate affinity chromatography. In vitro characterization of the CrmA-mutant protein revealed that under conditions where CrmA bound and

inhibited ICE, the mutant protein neither bound ICE (FIG. **3**B) nor inhibited its proteolytic activity (FIG. **3**A). The tertiary structure of CrmA-mutant, however, was not significantly altered by the point mutation, as its conformational signature was indistinguishable from that of wild-type CrmA on transferase urea gradient PAGE (FIG. **3**C), a method used to probe the tertiary structures of serpins (Goldenberg (1989) supra; Mast et al. (1991) supra; and Komiyama et al. (1994) supra).

Using these purified recombinant proteins, CrmA markedly inhibited the cleavage of PARP by Yama in vitro (FIG. **4**). When CrmA was replaced with an equivalent amount of CrmA-mutant protein, no inhibition of PARP cleavage was observed (FIG. **4**), indicting that the effect of CrmA was a function specifically of its ability to act as a protease inhibitor.

To confirm that CrmA directly interacts with Yama, the ability of either Pro-Yama or activated Yama to form a complex with either native CrmA or mutant CrmA was examined. Pro-Yama or activated Yama (both labeled with [35]-Met) were each incubated with either native CrmA or an equivalent amount of mutant CrmA recombinant protein. Each reaction was subjected to immunoprecipitation analysis using a polyclonal CrmA antiserum and the immunoprecipitates resolved by SDS-PAGE and visualized by phosphorimaging. CrmA associated with the activated two-subunit form of Yama but not with Pro-Yama (FIG. **5**B). CrmA-mutant bound neither activated Yama nor Pro-Yama (FIG. **5**B). It was determined that CrmA interacts directly with activated Yama to form an inhibitory complex.

Since CrmA inhibited the cleavage of PARP by Yama in vitro, it was inferred that if Yama is indeed responsible for PARP cleavage during apoptosis, then CrmA should inhibit PARP cleavage in vivo. To investigate this, the MCF7 breast carcinoma and BJAB lymphoma cell lines stably transfected with either vector were utilized, CrmA or CrmA-mutant expression constructs. Clonal cell lines which expressed the indicated proteins were selected and protein expression confirmed by immunoblotting using an anti-CrmA polyclonal antiserum (FIG. **6**A). In keeping with the in vitro findings, expression of CrmA inhibited proteolytic cleavage of PARP to the signature 85 kDa fragment normally generated during apoptosis induced by either Fas (BJAB cells) or TNF receptors (MCF7 cells), whereas in the vector ad CrmA-mutant lines, cleavage of PARP proceed unabated (FIG. **6**B, **6**C).

To investigate whether the in vivo blockage of PARP cleavage by CrmA and lack thereof by CrmA-mutant correlated with the ability of these proteins to inhibit apoptosis, the BJAB and MCF7 transfectants were examined. CrmA afforded protection from TNF-induced apoptosis as expected, whereas CrmA-mutant expressing lines showed no protection and were as sensitive as a vector-transfected line (FIG. **7**, bottom). The protection conferred by CrmA and lack thereof by CrmA -mutant was readily apparent on examination of nuclear morphology of propidium-iodide stained cells (FIG. **7**, top). When BJAB transfectants were examined for sensitivity to Fas-induced PCD, CrmA was protective whereas CrmA-mutant expression lines remained as sensitive as a vector control line (FIG. **7**, bottom). Thus, it was concluded that the divergent abilities of CrmA and CrmA-mutant to block PARP cleavage correlate with the abilities of these proteins to block cell death. The finding that CrmA inhibits the proteolytic activity of Yama (FIG. **4**) is significant, as it shows that the well-documented ability of CrmA to inhibit apoptosis can now be explained by its inhibition of Yama. Also, the finding that CrmA inhibits PARP cleavage in vivo is consistent with its inhibiting Yama.

EXPERIMENT NO. 6

To deterrnine whether cleavage of U1-70 kDa is an event characteristic of either Fas or TNF-R-induced apoptosis, the following were constructed.

Treatment with Anti-Fas Antibody or TNF and Preparation of Cell Lysates—MCF7 cells or derived transfectants were plated in 100-mM dishes at a concentration of 2×10^6 cells/dish. On the second day, cells were treated with TNF at 40 ng/ml for the indicated time periods. Following a PBS rinse, cells were harvested by scraping into 15 ml of PBS plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml aproptinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepsytatin), recovered by centrifugation, and lysed in 2.5 ml of sample buffer (50 mM Tri^5-HCl, pH 6.8,6 mM urea, 6% 2-mercaptoethanol, 3% SDS, and 0.003% bromphenol blue). In cases where nonadherent cells were present in the culture medium (e.g. at later time points), floating cells also were harvested by centrifugation and combined with the adherent cell pellet before lysis in sample buffer. BJAB cells or derived transfectants were aliquoted at a concentration of 5×10^5/ml into six-well dishes, with 4 ml in each well. The following day, cells were treated with anti-Fas antibody (250 ng/ml) for the indicated time periods, harvested by centrifugation, washed once with PBS plus protease inhibitors, and lysed in 2 ml of sample buffer. (FIGS. **17**A and **17**B).

Western Blotting-Immunoblotting to assess the state of U1-70 kDa was carried out similarly as described in Casciola-Rosen, L. A., et al. (1994) *J. Biol. Chem.*, 269:30757–30760. In brief, cell lysates (20 μl) were resolved by SDS-polyacrylamide gel electrophrophoresis and transferred to nitrocellulose membrane (Schleicher and Schnell) by electroblotting. Blots were blocked at room temperature for 1 hour using blocking buffer (10 mM Tris (pH 7.6), 150 mM NaCl, 0.5% Nonidet P-40, 3% bovine serum albumin). The U1-70 kD affinity purified antiserum was used at a 1:5000 dilution in blocking buffer and was incubated for 1 hour at room temperature. The secondary reagent, a biotinylated goat anti-human IgG antibody (Southern Biotech), was used at a dilution of 1:6700 in blocking buffer and was incubated for 50 minutes at room temperature. The tertiary reagent, a streptavidin-horseradish peroxidase conjugate (Southern Biotech), was used at a dilution of 1:10,000 in blocking buffer and was incubated at 30 minutes at room temperature. Visualization of signal was by electrochemiluminesence (Amersham Corp.).

EXPERIMENT NO. 7

Yama is Activated by Various Apoptotic Stimuli. Jurkat T cells expressed the 32 kDa Pro-Yama and treatment with staurosporine or anti-APO-1 antibody generated active 17 kDa and 12 kDa subunits, designated p20 and p11 herein. (FIG. **20**). Intermediate forms of the larger 17 kDa subunit were also observed and likely contain the small pro-domain of Yama (FIG. **20**).

EXPERIMENT NO. 8

Bcl-2 and Bcl-xL Functions Upstream of Yama—The bcl-2 family of proteins has been shown to prevent cell death induced by a variety of signals and in numerous cell types. However, little is known about where bcl-2 functions relative to the apoptotic proteases. To address this important question, Jurkat cells overexpressing bcl-2 or bcl-XL were constructed using episomal expression constructs. Expression of bcl-2 and bcl-XL in the pooled population was

confirmed by immunoblotting. (FIG. **21**A) Staurosporine has been shown to reliably induce bcl-2-inhibitable cell death in a variety of cell lines. As expected, Jurkat cells expressing bcl-2 and bcl-XL were resistant to staurosporine-induced apoptosis as assessed by DNA fragmentation (FIG. **21**B) and nuclear morphology. An apoptotic stimulus that is not consistently blocked by bcl-2 is activation of Fas/APO-1. In this Jurkat cell system, expression of bcl-2 or bcl-XL did not inhibit anti-APO-1-induced cell death or PARP cleavage. (FIGS. **21**B and C). Overexpression of bcl-2 or bcl-XL abrogated staurosporine-induced generation of actived Yama. (FIG. **21**D). Similar results were obtained using another bcl-2/bcl-XL-inhibitable death stimulus, thapsigargin, which is a specific inhibitor of the endoplasmic reticulum $Ca2^{+}$-ATPase. The inhibitory action of bcl-2 and bcl-xL could be "bypassed" by treatment with anti-APO-1 antibody, resulting in activation of the apoptotic proteases (FIG. **21**D). This result also was seen using a well characterized bcl-2 expressing CEM cell line (See Martin (1995) *Cell Death and Diff.*, 2:253–257).

Evidence for a Proteolytic Cascade in the FaslAPO-1 Cell Death Pathway- Unlike staurosporine and thapsigargin treatment, Fas/APO-1 induced Yama activation and resulting apoptosis was not blocked by bcl-2 or bcl-XL in this Jurkat cell system, CrmA expressing Jurkat cells were resistant to anti-APO-1-induced cell death, but not staurosporine-induced apoptosis (FIG. **22**B). Integrity of PARP was also monitored and, likewise, CrmA blocked PARP cleavage induced by Fas/APO-1 but not by staurosporine. To determine whether CrmA directly inhibited Yama in vivo, activation of the apoptotic protease was directly assessed. Consistent with DNA fragmentation and PARP analysis (FIGS. **22**B and **22**C), staurosporine-induced Yama activation was not blocked by CrmA (FIG. **22**D).

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 14

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GGCACGAGCG GATGGGTGCT ATTGTGAGGC GGTTGTAGAA GAGTTTCGTG AGTGCTCGCA      60

GCTCATACCT GTGGCTGTGT ATCCGTGGCC ACAGCTGGTT GGCGTCGCCT TGAAATCCCA     120

GGCCGTGAGG AGTTAGCGAG CCCTGCTCAC ACTCGGCGCT CTGGTTTTCG GTGGGTGTGC     180

CCTGCACCTG CCTCTTCCCG CATTCTCATT AATAAAGGTA TCC ATG GAG AAC ACT       235
                                               Met Glu Asn Thr
                                                 1

GAA AAC TCA GTG GAT TCA AAA TCC ATT AAA AAT TTG GAA CCA AAG ATC       283
Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu Glu Pro Lys Ile
  5                  10                  15                  20

ATA CAT GGA AGC GAA TCA ATG GAC TCT GGA ATA TCC CTG GAC AAC AGT       331
Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser Leu Asp Asn Ser
                 25                  30                  35

TAT AAA ATG GAT TAT CCT GAG ATG GGT TTA TGT ATA ATA ATT AAT AAT       379
Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile Ile Ile Asn Asn
             40                  45                  50

AAG AAT TTT CAT AAA AGC ACT GGA ATG ACA TCT CGG TCT GGT ACA GAT       427
Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg Ser Gly Thr Asp
         55                  60                  65

GTC GAT GCA GCA AAC CTC AGG GAA ACA TTC AGA AAC TTG AAA TAT GAA       475
Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn Leu Lys Tyr Glu
     70                  75                  80

GTC AGG AAT AAA AAT GAT CTT ACA CGT GAA GAA ATT GTG GAA TTG ATG       523
Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile Val Glu Leu Met
 85                  90                  95                  100

CGT GAT GTT TCT AAA GAA GAT CAC AGC AAA AGG AGC AGT TTT GTT TGT       571
Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser Ser Phe Val Cys
                105                 110                 115

GTG CTT CTG AGC CAT GGT GAA GAA GGA ATA ATT TTT GGA ACA AAT GGA       619
Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe Gly Thr Asn Gly
            120                 125                 130

CCT GTT GAC CTG AAA AAA ATA ACA AAC TTT TTC AGA GGG GAT CGT TGT       667
Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg Gly Asp Arg Cys
        135                 140                 145

AGA AGT CTA ACT GGA AAA CCC AAA CTT TTC ATT ATT CAG GCC TGC CGT       715
Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile Gln Ala Cys Arg
    150                 155                 160

GGT ACA GAA CTG GAC TGT GGC ATT GAG ACA GAC AGT GGT GTT GAT GAT       763
Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser Gly Val Asp Asp
165                 170                 175                 180

GAC ATG GCG TGT CAT AAA ATA CCA GTG GAG GCC GAC TTC TTG TAT GCA       811
Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp Phe Leu Tyr Ala
                185                 190                 195

TAC TCC ACA GCA CCT GGT TAT TAT TCT TGG CGA AAT TCA AAG GAT GGC       859
Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn Ser Lys Asp Gly
            200                 205                 210

TCC TGG TTC ATC CAG TCG CTT TGT GCC ATG CTG AAA CAG TAT GCC GAC       907
Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys Gln Tyr Ala Asp
        215                 220                 225

AAG CTT GAA TTT ATG CAC ATT CTT ACC CGG GTT AAC CGA AAG GTG GCA       955
Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn Arg Lys Val Ala
    230                 235                 240

ACA GAA TTT GAG TCC TTT TCC TTT GAC GCT ACT TTT CAT GCA AAG AAA      1003
Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe His Ala Lys Lys
245                 250                 255                 260
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CAG ATT CCA TGT ATT GTT TCC ATG CTC ACA AAA GAA CTC TAT TTT TAT     1051
Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Tyr
                265                 270                 275

CAC TAAAGAAATG GTTGGTTGGT GGTTTTTTTT AGTTTGTATG CCAAGTGAGA         1104
His

AGATGGTATA TTTGGTACTG TATTTCCCTC TCATTTGGGC CTACTCTCAT GCTG         1158
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
  1               5                  10                  15

Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
             20                  25                  30

Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
         35                  40                  45

Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
     50                  55                  60

Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
 65                  70                  75                  80

Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
                 85                  90                  95

Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
            100                 105                 110

Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
        115                 120                 125

Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
    130                 135                 140

Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145                 150                 155                 160

Gln Ala Cys Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
                165                 170                 175

Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
            180                 185                 190

Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
        195                 200                 205

Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
    210                 215                 220

Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225                 230                 235                 240

Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
                245                 250                 255

His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
            260                 265                 270

Leu Tyr Phe Tyr His
        275
```

(2) INFORMATION FOR SEQ ID NO:3:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /label= CrmA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Tyr Thr Glu Ala Ala Ala Ala Thr Cys Ala Leu Val Ala Asp
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..14
(D) OTHER INFORMATION: /label= CrmA-mutant

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /note= “Altered from Thr to Arg by site-directed mutagenesis”

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Tyr Arg Glu Ala Ala Ala Ala Thr Cys Ala Leu Val Ala Asp
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1468 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 295..1317

(ix) FEATURE:
(A) NAME/KEY: mutation
(B) LOCATION: replace(1165..1167, “”)
(D) OTHER INFORMATION: /note= “coding region altered to change amino acid from Thr to Arg for mutant CrmA gene. ”

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
TCCATGGAAG AACGAAAGTA GTATAAAAGT AATAAAACAA AAAAAAGAAT ATAAAAAATT     60

TATAGCCACT TTCTTTGAGG ACTGTTTTCC TGAAGGAAAT GAACCTCTGG AATTAGTTAG    120

ATATATAGAA TTAGTATACA CGCTAGATTA TTCTCAAACT CCTAATTATG ACAGACTACG    180

TAGACTGTTT ATACAAGATT GAAAATATAT TTCTTTTTAT TGAGTGGTGG TAGTTACGGA    240

TATCTAATAT TAATATTAGA CTATCTCTAT CGTCACACAA CAAAATCGAT TGCC ATG      297
                                                          Met

GAT ATC TTC AGG GAA ATC GCA TCT TCT ATG AAA GGA GAG AAT GTA TTC      345
Asp Ile Phe Arg Glu Ile Ala Ser Ser Met Lys Gly Glu Asn Val Phe
    280                 285                 290

ATT TCT CCA CCG TCA ATC TCG TCA GTA TTG ACA ATA CTG TAT TAT GGA      393
Ile Ser Pro Pro Ser Ile Ser Ser Val Leu Thr Ile Leu Tyr Tyr Gly
```

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295                 300                 305                 310

GCT AAT GGA TCC ACT GCT GAA CAG CTA TCA AAA TAT GTA GAA AAG GAG      441
Ala Asn Gly Ser Thr Ala Glu Gln Leu Ser Lys Tyr Val Glu Lys Glu
                315                 320                 325

GCG GAC AAG AAT AAG GAT GAT ATC TCA TTC AAG TCC ATG AAT AAA GTA      489
Ala Asp Lys Asn Lys Asp Asp Ile Ser Phe Lys Ser Met Asn Lys Val
            330                 335                 340

TAT GGG CGA TAT TCT GCA GTG TTT AAA GAT TCC TTT TTG AGA AAA ATT      537
Tyr Gly Arg Tyr Ser Ala Val Phe Lys Asp Ser Phe Leu Arg Lys Ile
        345                 350                 355

GGA GAT AAT TTC CAA ACT GTT GAC TTC ACT GAT TGT CGC ACT GTA GAT      585
Gly Asp Asn Phe Gln Thr Val Asp Phe Thr Asp Cys Arg Thr Val Asp
    360                 365                 370

GCG ATC AAC AAG TGT GTT GAT ATC TTC ACT GAG GGG AAA ATT AAT CCA      633
Ala Ile Asn Lys Cys Val Asp Ile Phe Thr Glu Gly Lys Ile Asn Pro
375                 380                 385                 390

CTA TTG GAT GAA CCA TTG TCT CCA GAT ACC TGT CTC CTA GCA ATT AGT      681
Leu Leu Asp Glu Pro Leu Ser Pro Asp Thr Cys Leu Leu Ala Ile Ser
                395                 400                 405

GCC GTA TAC TTT AAA GCA AAA TGG TTG ATG CCA TTT GAA AAG GAA TTT      729
Ala Val Tyr Phe Lys Ala Lys Trp Leu Met Pro Phe Glu Lys Glu Phe
            410                 415                 420

ACC AGT GAT TAT CCC TTT TAC GTA TCT CCA ACG GAA ATG GTA GAT GTA      777
Thr Ser Asp Tyr Pro Phe Tyr Val Ser Pro Thr Glu Met Val Asp Val
        425                 430                 435

AGT ATG ATG TCT ATG TAC GGC GAG GCA TTT AAT CAC GCA TCT GTA AAA      825
Ser Met Met Ser Met Tyr Gly Glu Ala Phe Asn His Ala Ser Val Lys
    440                 445                 450

GAA TCA TTC GGC AAC TTT TCA ATC ATA GAA CTG CCA TAT GTT GGA GAT      873
Glu Ser Phe Gly Asn Phe Ser Ile Ile Glu Leu Pro Tyr Val Gly Asp
455                 460                 465                 470

ACT AGT ATG GTG GTA ATT CTT CCA GAC AAT ATT GAT GGA CTA GAA TCC      921
Thr Ser Met Val Val Ile Leu Pro Asp Asn Ile Asp Gly Leu Glu Ser
                475                 480                 485

ATA GAA CAA AAT CTA ACA GAT ACA AAT TTT AAG AAA TGG TGT GAC TCT      969
Ile Glu Gln Asn Leu Thr Asp Thr Asn Phe Lys Lys Trp Cys Asp Ser
            490                 495                 500

ATG GAT GCT ATG TTT ATC GAT GTG CAC ATT CCC AAG TTT AAG GTA ACA     1017
Met Asp Ala Met Phe Ile Asp Val His Ile Pro Lys Phe Lys Val Thr
        505                 510                 515

GGC TCG TAT AAT CTG GTG GAT GCG CTA GTA AAG TTG GGA CTG ACA GAG     1065
Gly Ser Tyr Asn Leu Val Asp Ala Leu Val Lys Leu Gly Leu Thr Glu
    520                 525                 530

GTG TTC GGT TCA ACT GGA GAT TAT AGC AAT ATG TGT AAT TCA GAT GTG     1113
Val Phe Gly Ser Thr Gly Asp Tyr Ser Asn Met Cys Asn Ser Asp Val
535                 540                 545                 550

AGT GTC GAC GCT ATG ATC CAC AAA ACG TAT ATA GAT GTC AAT GAA GAG     1161
Ser Val Asp Ala Met Ile His Lys Thr Tyr Ile Asp Val Asn Glu Glu
                555                 560                 565

TAT ACA GAA GCA GCT GCA GCA ACT TGT GCG CTG GTG GCA GAC TGT GCA     1209
Tyr Thr Glu Ala Ala Ala Ala Thr Cys Ala Leu Val Ala Asp Cys Ala
            570                 575                 580

TCA ACA GTT ACA AAT GAG TTC TGT GCA GAT CAT CCG TTC ATC TAT GTG     1257
Ser Thr Val Thr Asn Glu Phe Cys Ala Asp His Pro Phe Ile Tyr Val
        585                 590                 595

ATT AGG CAT GTC GAT GGC AAA ATT CTT TTC GTT GGT AGA TAT TGC TCT     1305
Ile Arg His Val Asp Gly Lys Ile Leu Phe Val Gly Arg Tyr Cys Ser
    600                 605                 610

CCA ACA ACT AAT TAAATCACAT TCTTAATATT AGAATATTAG AATATTATAT         1357
```

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Pro Thr Thr Asn
615

AGTTAAGATT TTTACTAATT GGTTAACCAT TTTTTTAAAA AAATAGAAAA AAAACATGTT 1417

ATATTAGCGA GGGTCGTTAT TCTTCCAATT GCAATTGGTA AGATGACGGC C 1468

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 341 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asp Ile Phe Arg Glu Ile Ala Ser Ser Met Lys Gly Glu Asn Val
1 5 10 15

Phe Ile Ser Pro Pro Ser Ile Ser Ser Val Leu Thr Ile Leu Tyr Tyr
20 25 30

Gly Ala Asn Gly Ser Thr Ala Glu Gln Leu Ser Lys Tyr Val Glu Lys
35 40 45

Glu Ala Asp Lys Asn Lys Asp Asp Ile Ser Phe Lys Ser Met Asn Lys
50 55 60

Val Tyr Gly Arg Tyr Ser Ala Val Phe Lys Asp Ser Phe Leu Arg Lys
65 70 75 80

Ile Gly Asp Asn Phe Gln Thr Val Asp Phe Thr Asp Cys Arg Thr Val
85 90 95

Asp Ala Ile Asn Lys Cys Val Asp Ile Phe Thr Glu Gly Lys Ile Asn
100 105 110

Pro Leu Leu Asp Glu Pro Leu Ser Pro Asp Thr Cys Leu Leu Ala Ile
115 120 125

Ser Ala Val Tyr Phe Lys Ala Lys Trp Leu Met Pro Phe Glu Lys Glu
130 135 140

Phe Thr Ser Asp Tyr Pro Phe Tyr Val Ser Pro Thr Glu Met Val Asp
145 150 155 160

Val Ser Met Met Ser Met Tyr Gly Glu Ala Phe Asn His Ala Ser Val
165 170 175

Lys Glu Ser Phe Gly Asn Phe Ser Ile Ile Glu Leu Pro Tyr Val Gly
180 185 190

Asp Thr Ser Met Val Val Ile Leu Pro Asp Asn Ile Asp Gly Leu Glu
195 200 205

Ser Ile Glu Gln Asn Leu Thr Asp Thr Asn Phe Lys Lys Trp Cys Asp
210 215 220

Ser Met Asp Ala Met Phe Ile Asp Val His Ile Pro Lys Phe Lys Val
225 230 235 240

Thr Gly Ser Tyr Asn Leu Val Asp Ala Leu Val Lys Leu Gly Leu Thr
245 250 255

Glu Val Phe Gly Ser Thr Gly Asp Tyr Ser Asn Met Cys Asn Ser Asp
260 265 270

Val Ser Val Asp Ala Met Ile His Lys Thr Tyr Ile Asp Val Asn Glu
275 280 285

Glu Tyr Thr Glu Ala Ala Ala Ala Thr Cys Ala Leu Val Ala Asp Cys
290 295 300

Ala Ser Thr Val Thr Asn Glu Phe Cys Ala Asp His Pro Phe Ile Tyr
305 310 315 320

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Val Ile Arg His Val Asp Gly Lys Ile Leu Phe Val Gly Arg Tyr Cys
325 330 335

Ser Pro Thr Thr Asn
340

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /note= “Mutant Cys to Met or Ala”

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Ala Cys Arg Gly
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACCGGAATT CCACCATGGA TATCTTCAGG GAAATCG 37

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTCTAGACT CGAGTTAATT AGTTGTTGGA GAGCAATATC 40

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTATGTTTA TCGATGTGCA CATTCCCAAG 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCACAAGTTG CTGCGGCTGC TTCGCGATAC TCTTCATTGA CATC 44

(2) INFORMATION FOR SEQ ID NO:12:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTCTAGACT CGAGTTAATT AGTTGTTGGA GAGCAATATC 40

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATGTCAATG AAGAGTATCG CGAAGCAGCC GCAGCAACTT GTGC 44

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /label= Boc-
/note= "Protecting group attached"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /note= "p-nitroanilide group attached"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Ala Pro Asp

What is claimed is:

1. A method for screening for an agent having ability to inhibit apoptosis in a cell as compared with cowpox cytokine response modified A (CrmA), comprising:

a) inducing apoptosis in a cell culture or tissue culture having a Fas, T cell receptor (TCR) or Tumor Necrosis Factor receptor (TNF-R) that mediates apoptosis;

b) dividing the apoptosis-induced cell culture or tissue culture into at least a first and a second sample;

c) contacting the first sample with an agent to be tested;

d) contating the second sample with a nucleic acid molecule coding for a gene product having cowpox cytokine response modified A (CrmA) biological activity or CrmA gene product under conditions to inhibit apoptosis in the control sample; and

e) assaying the samples of steps c) and d) for apoptosis, wherein the absence of apoptosis in the test sample indicates that the agent to be tested is an inhibitor of apoptosis.

2. The method according to claim **1** wherein step e) comprises assaying for the cleavage of poly (ADP ribose) polymerase (PARP) protein.

3. The method according to claim **1** wherein step e) comprises assaying for the cleavage of the 70 kDa protein of the U1 small ribonucleoprotein particle (U1-70) kDA protein.

4. The method of claim **1**, wherein the agent to be tested is a candidate for inhibiting Fas-induced apoptosis.

5. Tbe method according to claim **2**, wherein the agent to be tested is a candidate for inhibiting Fas-induced apoptosis.

6. The method of claim **3**, wherein the agent to be tested is a candidate for inhibiting Fas-induced apoptosis.

7. A cell-free in vitro method for screening for candidate agents having a biological function in an apoptotic pathway that is inhibitable by a cowpox cytokine response modified A (CrmA) gene or gene product, comprising incubating an effective amount of the 70 kDa protein of the U1 small ribonucleoprotein particle (U1-70) with an apoptosis-activating agent and a test agent under suitable conditions to cleave U1-70 to the 40 kDa form and assaying to determine if cleavage of U1-70 occurred, wherein the absence of the 40 kDa form indicates that the test agent is a candidate for inhibiting the apoptotic pathway.

* * * * *